STRUCTURE AND FUNCTION OF HEDGEHOG ACYLTRANSFERASE IN NORMAL AND CANCER CELLS

by

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DEDICATION

To my parents, Azniv and Achot, for showing me how to lead a meaningful and joyous life. To my siblings, Ara and Anouch, for always being by my side in this journey.

ABSTRACT

Hedgehog acyltransferase (Hhat) is a multipass transmembrane enzyme that mediates the covalent attachment of the 16-carbon fatty acid palmitate to the N-terminal cysteine of Sonic Hedgehog (Shh). Palmitoylation of Shh by Hhat is critical for short and long range signaling. The Shh signaling pathway has been implicated in the progression of breast cancer. To determine the functional significance of Hhat expression in breast cancer, we used a panel of estrogen receptor (ER) positive and negative cell lines. Here we show that Hhat is a novel target for inhibition of ER positive, HER2 amplified, and tamoxifen resistant breast cancer cell growth. Depletion of Hhat with lentiviral shRNA decreased both anchorage-dependent and anchorage-independent proliferation of ER positive, but not triple negative, breast cancer cells. Treatment with RU-SKI 43, a small molecule inhibitor of Hhat recently identified by our group, also reduced ER positive cell proliferation. Overexpression of Hhat in ER positive cells not only rescued the growth defect in the presence of RU-SKI 43 but also resulted in increased cell proliferation in the absence of drug. Furthermore, depletion or inhibition of Hhat also reduced proliferation of HER2 amplified as well as tamoxifen resistant cells. Moreover, Hhat regulated the proliferation of both Shh responsive and non-responsive ER positive cells, suggesting a Shh independent function for Hhat. Together, these data suggest that Hhat plays a critical role in ER positive, HER2 amplified, and hormone resistant breast cancer proliferation and highlights the potential promise of Hhat inhibitors for therapeutic benefit.

To enhance our understanding of Hhat structure and function, we also conducted a comprehensive analysis of its transmembrane topology. Bioinformatics analysis of

transmembrane domains within human Hhat using ten different algorithms resulted in highly consistent predictions in the C-terminal, but not in the N-terminal, region of Hhat. To empirically determine the topology of Hhat, we designed and exploited Hhat constructs containing either terminal or 12 different internal epitope tags. We used selective permeabilization coupled with immunofluorescence as well as a protease protection assay to demonstrate that Hhat contains ten transmembrane domains and two re-entrant loops. The invariant His and highly conserved Asp residues within the membrane bound O-acyltransferase (MBOAT) homology domain are segregated on opposite sides of the endoplasmic reticulum membrane. The localization of His379 on the lumenal membrane surface is consistent with a role for this invariant residue in catalysis. Analysis of the activity and stability of the Hhat constructs revealed that the C-terminal MBOAT domain is especially sensitive to manipulation. Moreover, there was remarkable similarity in the overall topological organization of Hhat and ghrelin O-acyltransferase, another MBOAT family member. Knowledge of the topological organization of Hhat could serve as an important tool for further design of selective Hhat inhibitors.

BIOGRAPHICAL SKETCH

Armine Matevossian was born in Yerevan, Armenia and moved to Chelmsford, MA at the age of ten. She attended University of Massachusetts in Amherst where she received a Bachelor of Science degree with high honors in Biochemistry and Molecular Chemistry. She entered the Gerstner Sloan Kettering Graduate School of Biomedical Sciences in 2008 and later joined the laboratory of Dr. Marilyn Resh. During her graduate career, Armine examined the function of Hhat in breast cancer and elucidated the topological organization of Hhat.

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LIST OF ABBREVIATIONS

ACAT: Acyl:CoA cholesterol-acyltransferase

ATO: Arsenic trioxide

BCC: Basal cell carcinoma

BOC: brother of CDO

BP: Benzophenone

βTrCP: β-transducing-repeat-containing protein

CDO: CAM-related/downregulated by oncogene

Ci: Cubitus interruptus

CitS: Citrate transporter

CkIα: Casein kinase Iα

CKI: Caseine kinase I

Cos2: Costal 2

CRD: Cysteine rich domain

CSC: Cancer stem cell

CTD: C-terminal domain

CUL1: Cullin 1

CYR61: Cysteine-rich angiogenic inducer 61

DCIS: Ductal carcinoma *in situ*

DGAT: Acyl:CoA diacylglycerol-acyltransferase

Dhh: Desert Hedgehog

DHHC-CRD: Asp-His-His-Cys- cysteine rich domain

Disp: Dispatched

Dpp: Decapentaplegic

DSD: Disorders of sex development

DRAL: downregulated in rhabdomyosarcoma LIM-domain protein

ER: Estrogen receptor

ESCRT: endosomal sorting complex required for transport

EVC: Ellis-van Creveld syndrome protein

EXT: exostosin glycosyltransferase

Fu: Fused

GAS1: Growth arrest-specific 1

GDNF: glial cell line-derived neurotrophic factor

GLI: Glioma-associated oncogene homolog

GNP: Granule neuron progenitors/precursors

GOAT: Ghrelin O-acyltransferase

GPC3: proteoglycan family member Glypican 3

GPCR: G-protein coupled receptors

GPI: glycosylphosphatidylinositol

Gprk2: G-protein coupled receptor kinase 2

GSK3β: Glycogen synthase kinase 3β

GST: Glutathione-S-transferase

GUP1: Glycerol uptake protein 1

HER2: Human epidermal growth factor

Hh: Hedgehog

Hhat: Hedgehog acyltransferase

Hhatl: Hhat-like

HhN: N-terminal domain of Hedgehog

HIP: Hedgehog interacting protein

HMVEC: Human umbilical vein endothelial

HPI: Hedgehog pathway inhibitors

HSC: Hedgehog signaling complex

HUVEC: Human cardiac microvascular endothelial

IDC: Invasive ductal carcinoma

ILC: Invasive lobular carcinoma

Ihh: Indian Hedgehog

Itch: Itchy E3 Ubiquitin Protein Ligase

KIF3A: Kinesin family member 3A

LPLAT: Lysophospholipid acyltransferase

LRP2: Lipoprotein receptor related protein

MB: Medulloblastoma

MBOAT: Membrane bound O-acyltransferase

MMTV: Mouse mammary tumor virus

mTMD: marginally hydrophobic transmembrane domain

NALP1: NLR family, pyr in domain containing 1

OPN: Osteopontin

OST: Oligosaccharyl trnasferase

PAT: Protein acyltransferases

PDI: Protein disulfide isomerase

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

Porcn: Porcupine

PR: Progesterone receptor

Ptch: Patched

Rasp: Raspberry

RL: re-entrant loop

RND: Resistance-Nodulation-Division

SILAC: stable isotope labeling by amino acids in cell culture

SFK: Src family kinase

SKP1: S phase associated protein kinase 1

Smo: Smoothened

Shh: Sonic Hedgehog

SPC: Signal peptidase complex

SSD: Sterol sensing domain

SuFu: Suppressor of Fused

TEB: Terminal end bud

TGFβ: Transforming growth factor β

TMD: Transmembrane domain

TRAM: Translocating chain-associated membrane protein

TRAP: Tetrameric translocon-associated protein

TUCAN: caspase recruitement (CAR)-domain containing proteins #8

TULP3: Tubby-related protein 3

WWP2: WW Domain Containing E3 Ubiquitin Protein Ligase 2

Chapter One

Introduction

Hedgehog (Hh) was identified by Nüsslein-Volhard and Wieschaus in a screen for genes required for establishing embryonic segment polarity in *Drosophila* [1]. Mutations in Hh led to the duplication of denticles and a loss of naked cuticle, resulting in a phenotype reminiscent of hedgehog spines. In the early 1990s, four groups independently reported that the *hh* gene encodes a putative secreted protein [2-5]. The subsequent discovery of Hh signaling in vertebrates established Hh as a critical, conserved regulator of embryogenesis [6-10]. It is now well established that Hh directs the proper development of diverse tissues including the central nervous system, hematopoietic system, gastrointestinal tract, lung, and heart [11]. Interestingly, while Hh expression is downregulated in most adult tissues, it is required for proper maintenance and function of stem cells. Furthermore, Hh signaling is aberrantly reactivated in numerous tumors [12, 13].

Many components of Hh signaling are conserved between *Drosophila* and mammals. However, the mammalian pathway is more complex and contains additional components as well as multiple orthologs of ligands, receptors, and signaling molecules. Canonical Hh signaling is initiated when Hh interacts with the transmembrane receptor Patched (Ptch). This relieves Ptch-mediated inhibition of the transducer Smoothened (Smo) and leads to the stabilization and nuclear translocation of the Gli (glioma-associated oncogene homolog) family of transcription factors [11]. The resulting activation of target gene transcription regulates various cellular processes such as cell fate determination, proliferation, and survival [11]. Hh is synthesized as a precursor protein that undergoes autoprocessing to produce a C-terminal fragment and an N-terminal fragment (HhN), which retains all signaling activity [14, 15]. HhN is modified with two lipids. Cholesterol is covalently attached to the C-terminus during the autoprocessing reaction [16]. Cholesterol attachment contributes to long-range signaling activity, but is not essential for signaling [17]. The N-terminus of HhN is modified by covalent attachment of the 16-carbon fatty acid palmitate at the N-terminal cysteine [18]. Hh palmitoylation is catalyzed by Hedgehog acyltransferase (Hhat), a multipass transmembrane enzyme that belongs to the membrane bound O-acyltransferase (MBOAT) family [19]. Palmitoylation of Hh is critical for signaling activity and Hhat activity is required for early embryonic development [17, 20-23]. In addition, we and others have recently demonstrated that Hhat activity is required for the proliferation of pancreatic cancer cells *in vivo* and for the maintenance of a stem-like phenotype in lung squamous cell carcinoma [24-27]. Here we 1) identify Hhat as a novel drug target in

specific subtypes of breast cancer, and 2) determine the topological organization of Hhat across the endoplasmic reticulum membrane.

The Hedgehog signaling pathway

In vertebrates, there are three Hh family members – Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) – which vary in their spatial and temporal expression patterns [28]. Shh is the most widely expressed and the best characterized of the three family members. Shh regulates the establishment of both the left-right and the dorsal-ventral axes in embryos [29-31]. Shh also controls patterning and development of diverse tissues including the central neural crest, smooth muscle, gastrointestinal tract, and lung [32-35]. Ihh is expressed in the primitive endoderm and Ihh deficiency is lethal due to defects in hematopoiesis and vasculogenesis [36]. In addition, Ihh is expressed in prehypertrophic chondrocytes in the growth plates of bones, and it regulates chondrocyte proliferation and osteoblast development [37, 38]. Dhh expression is confined mostly to gonads and Dhh deficient mice are viable. In males, Dhh expression in Sertoli cells of the testis induces Leydig cell differentiation. This process is required for spermatogenesis; therefore, Dhh deficiency causes infertility in males [39, 40]. In females, the granulosa cells of ovaries secrete both Ihh and Dhh which mediate the development of theca cells [41].

Canonical Shh signaling is initiated when Shh interacts with its receptor Ptch, a 12-pass transmembrane protein (Fig. 1.1). Genetic analysis established that Ptch acts downstream of Hh [42] and *in vitro* studies showed that purified Shh can bind to the

extracellular domains of Ptch [43-45]. Mutational analyses established that distinct domains of Ptch are required for Shh binding and for Smo inhibition [46, 47]. Ptch is a member of the Resistance-Nodulation-Division (RND) family of prokaryotic permeases [48, 49] and contains a sterol sensing domain (SSD). Individual mutation of various residues in the SSD domain abolish Ptch-mediated Smo repression without altering binding or internalization of Hh [50, 51]. Mutations within the RND also lead to reduced inhibition of Smo [52]. Upon binding to Ptch, Hh is apparently rapidly internalized by target cells as it appears mainly in vesicular structures [53] as well as lysosomes in certain cell types [54]. Internalization and degradation of the ligand-receptor complex attenuates pathway activation. The response to Shh has been proposed to be regulated either by the ratio of ligand bound or unbound Ptch or the absolute amount of unbound Ptch [52, 55, 56].

Vertebrates have two Ptch isoforms, Ptch1 and Ptch2, which share significant structural similarities and can bind all three Hh ligands [57]. Ptch1 is widely expressed and Ptch1 deficient mice die at embryonic day 9, due to abnormal heart development [58]. In addition, $Ptch1^{-/-}$ mice have defects in neural tube and limb development. Transgenic expression of Ptch in $Ptch1^{-/-}$ mice can rescue, in part, these phenotypes [59]. Ptch2 is also expressed in various tissues including brain, stomach, heart, and liver [57]. Interestingly, Ptch2 is highly expressed in testis and in peripheral nerve cells, where Dhh expression is also high [57, 60]. These findings suggest that Ptch2 serves as the main receptor for Dhh. Despite its broad expression pattern, Ptch2 deficiency is not lethal. However, $Ptch2^{-/-}$ mice develop skin defects such as loss of hair follicle structures and epidermal hyperplasia [61].

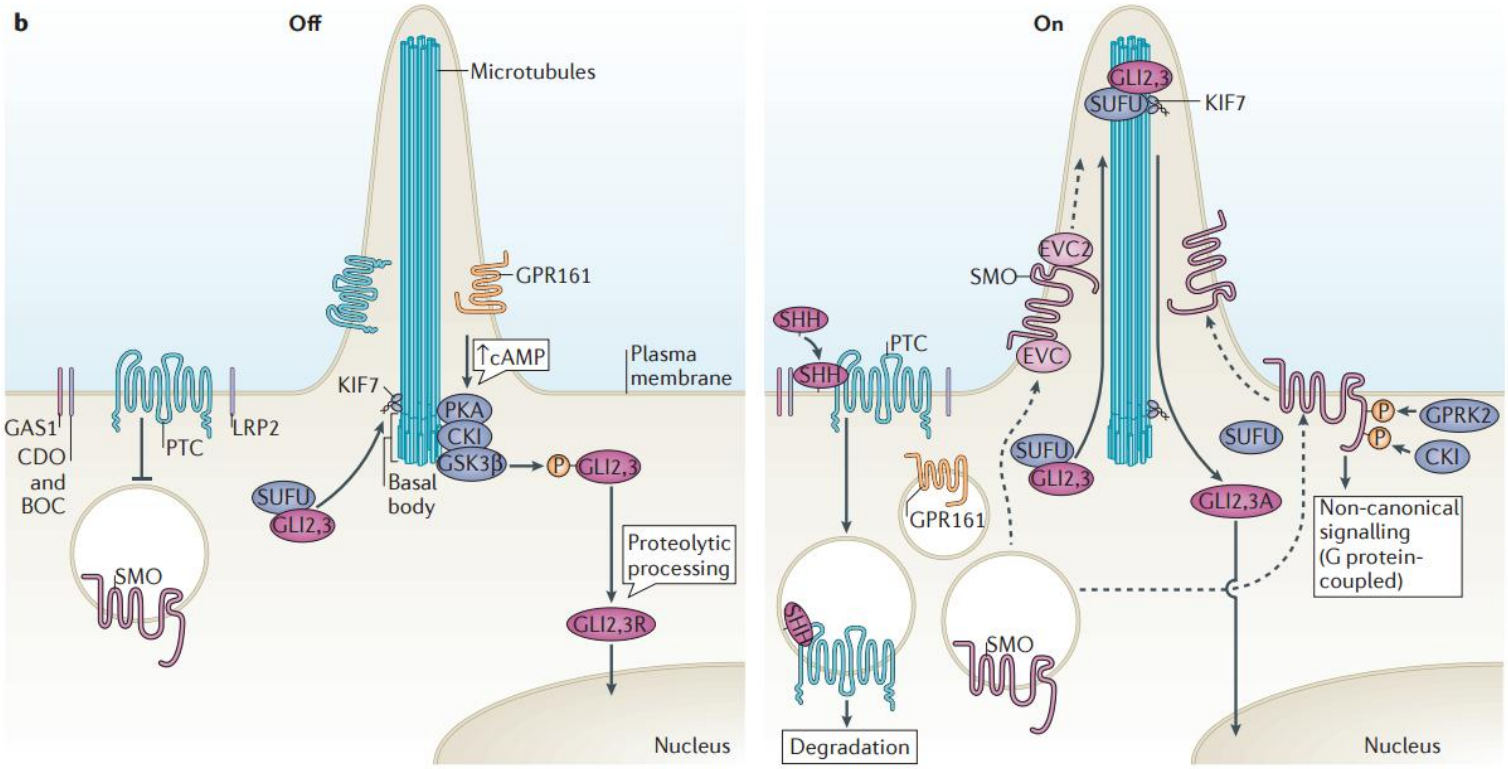

Figure 1.1 The canonical Hedgehog signaling pathway in vertebrates. *Left panel,* In the absence of HH ligands, PTCH, accumulated in and around the primary cilium, prevents SMO from entering the cilium. At the base of the cilium, GLI2 and GLI3 are phosphorylated by PKA, CKI and GSK3β, which induces proteolytic prossesing to generate the GLI2R and GLI3R repressor forms. *Right panel,* In the presence of HH ligands, the PTCH-HH complex is internalized, allowing SMO to translocate into the cilia and associate with EVC1/2 and KIF3A. Activation of SMO leads to increased dissociation of the SUFU-GLI2/3 complex, allowing the full-length GLI2A and GLI3A activator forms to translocate into the nucleus and initiate target gene transcription. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, 14, 416-29, Copyright 2013. http://www.ncbi.nlm.nih.gov/pubmed/?term=23719536

In addition to Ptch, three co-receptors promote Hh-Ptch binding: growth arrest-specific 1 (GAS1), CAM-related/downregulated by oncogene (CDO), and brother of CDO (BOC) (Fig. 1.1) [62-66]. These receptors form distinct multi-molecular complexes with Ptch and facilitate Hh binding to Ptch [67]. GAS1 is unique to vertebrates whereas CDO and BOC are conserved from *Drosophila* to mouse [68]. GAS1 is a glycosylphosphatidylinositol (GPI) anchored protein that shares homology with the glial cell line-derived neurotrophic factor (GDNF) receptor [69-71]. CDO and BOC are structurally related integral membrane proteins that contain an extracellular domain with a series of immunoglobin and fibronectin-like repeats [64, 65, 72]. Interestingly, different fibronectin repeats are required for binding *Drosophila* Hh and mammalian Shh [72]. Genetic deletion of only one receptor has little to no effect on Shh signaling during development whereas genetic ablation of all three co-receptors leads to near complete abrogation of Hh signaling *in vivo* [62, 68]. The requirement for each co-receptor seems to be tissue specific. For example, CDO and BOC double knockout mice show defects in Shh signaling in the developing neural tube but not the developing limb. Instead, in the limb, digit specification requires the expression of GAS1 and BOC [68].

Genetic evidence suggests that Hh ligand interaction with glypicans further modulates signaling. Hh transport is disrupted if neighboring cells lack heparan sulfate synthesizing enzymes of the EXT (exostosin glycosyltransferase) family [73-75]. Additionally, Shh seems to bind heparan and heparan sulfate chains on proteoglypicans on the cell surface and these interactions promote optimal signaling [76-78]. In particular, Hh binding to a heparan sulfate proteoglycan family member Glypican 3 (GPC3) restricts Hh transport [79]. Vertebrates, but not *Drosophila*, also express the Hh interacting

protein (HIP), which binds to and curtails the spread of Hh ligands through tissues [34, 80, 81].

Shh interaction with the receptor complex relieves Ptch mediated inhibition of Smo (Fig. 1.1) [30, 82-84]. Smo has seven-transmembrane domains and is structurally similar to G-protein coupled receptors (GPCRs) [83, 84]. A few studies suggest that Smo can also interact with heterotrimeric GTP binding regulatory proteins (G proteins). Smo can activate all members of the G_i family leading to transcriptional activation of Gli [85-87]. This signal alone, however, is not sufficient to activate Gli, suggesting there are at least two inputs from Smo to Gli [85]. Smo contains a highly conserved extracellular N-terminal cysteine-rich domain (CRD) and a cytoplasmic C-terminal domain (CTD) [88, 89]. Mutations in either CRD or CTD disrupt Smo activity [84, 89]. However, the structure of the C-terminal cytoplasmic tails of Smo is not conserved across species. While the mouse and zebrafish tails of Smo are very similar, they share only a weak similarity with that of *Drosophila*. The C-terminal tails of *Drosophila* Smo mediate binding to Costal 2 (Cos2), suggesting the interaction between Smo and Cos2 is not conserved in vertebrates [90].

The mechanism of Ptch-mediated Smo inhibition has yet to be determined. However, studies suggest either a transient interaction or action through a distance because the two proteins do not co-localize and Ptch can inhibit Smo at a stoichiometry up to 1:50 [52]. The presence of an RND permease motif in Ptch has led to suggestions that Ptch transports small, hydrophobic molecules across the plasma membrane that either agonize or antagonize Smo [52]. Consistent with this hypothesis, natural products, including the steroidal alkaloid cyclopamine, and synthesized small molecules can

regulate Smo activity [91]. In addition, Smo can directly bind oxysterols, oxygenated derivates of cholesterol, which can also activate Hh signaling [92-94]. To date, there is no evidence that Ptch functions in oxysterol transport. However, Ptch can bind cholesterol and one study suggests that, when overexpressed, Ptch can promote the efflux of a cholesterol derivative [95].

While the precise mechanism of Ptch-mediated Smo inhibition remains unknown, it is clear that subcellular localization plays a critical role in regulating Hh signal transduction. In *Drosophila*, Hh pathway activation coincides with cell surface accumulation of Smo [89, 96, 97]. During Smo activation, the cytoplasmic tails of Smo dimers undergo a conformational switch from a closed to an open configuration [98-100]. This switch is induced by sequential or gradual phosphorylation of its cytoplasmic tails by protein kinase A (PKA), casein kinase Iα (CkIα), CkII, and Gprk2 (G-protein coupled receptor kinase 2) in *Drosophila* [101-103]. However, the residues that are phosphorylated are not conserved, and genetic analyses show that loss of PKA activity in mice does not alter Smo trafficking [104]. Mammalian Smo is regulated by CKIα and GPRK2 and seems to be internalized upon Hh pathway activation [105, 106].

A number of genetic studies elucidated the importance of cilia in Hh signaling [90, 107]. In the absence of ligand, Ptch is localized in and around the primary cilia, while Smo is present at low levels [108]. Upon ligand binding, Ptch translocates out of the cilia and Smo molecules accumulate in the cilia either from intracellular vesicles or the plasma membrane (Fig. 1.1) [96, 109, 110]. Activated Smo can then interact with β-arrestin and KIF3A (Kinesin Family Member 3A), which facilitates Smo accumulation in cilia [111, 112]. Furthermore, Hh signal transduction also requires the interaction of

activated Smo with the ciliary proteins Ellis-van Creveld syndrome protein (EVC) and EVC2 [113].

Activation of Smo initiates a signaling cascade culminating in the stabilization and nuclear translocation of the Ci/GLI family transcription factors and initiation of target gene transcription (Fig. 1.1). In *Drosophila*, the transcriptional output of Hh signaling is mediated by one ancestral gene encoding Cubitus interruptus (Ci). In vertebrates, there are three related family members (Glioma-Associated Oncogene Homolog (GLI) 1, GLI2, and GLI3 [114, 115]. Ci and GLI proteins are highly homologous only in the zinc finger DNA binding domain [116].

Ci, GLI2 and GLI3 contain both a C-terminal activation and an N-terminal repressor domain. In *Drosophila*, Hh signaling regulates whether Ci acts as a repressor or activator. In vertebrates, GLI2 acts primarily as a transcriptional activator and GLI2 knockouts are embryonic lethal [117-119]. GLI3, on the other hand, acts mostly to repress gene transcription. GLI1 is unique in that it only contains an activator domain and is not required to initiate Shh signaling. Instead, GLI1 amplifies the transcriptional response to Shh signaling. Accordingly, GLI1 function is dispensable for normal development in mammals [120]. Interestingly, GLI1 is essential for Hh signaling in zebrafish [121].

The balance between repressive and activating functions of Ci/GLI is regulated by the strength and duration of Hh signaling. Pathway activation can regulate gene transcription by targeting activator GLI proteins to target genes, which may act by displacing repressor forms of GLI from enhancer regions [13, 122]. In mammals, target genes include components of the Hh pathway: GLI-1, PTCH1, and hHIP. Increased

transcription of both Ptch and HIP establishes a ligand-dependent negative feedback loop to attenuate pathway activation. Additionally, GLI1 and GLI2 regulate transcription of genes involved in cellular proliferation (CCND1/2, MYCN, SPP1, BMI-1, FOXM1), survival (BCL2, MDM2), and epithelial-mesenchymal transition (BMP1, MUC5AC, JAG2) [123]. Some genes are regulated globally whereas others exhibit a tissue specific pattern of expression [124].

The regulation of Ci or GLI proteins in response to Hh is quite similar in invertebrates and vertebrates; however, important differences do exist [90]. In both, whether Ci/GLI function as activators or repressors of the pathway depends on a series of phosphorylation events. In *Drosophila*, Ci is linked to its regulator kinases in the Hh signaling complex (HSC) [125]. In cultured cells, the kinesin-like protein Cos2 transports the HSC along microtubules [126]. Cos2 not only acts as a scaffold between Ci and its regulators but also directly interacts with Smo [88, 127-129]. HSC movement along microtubules is apparently required for proper regulation of Ci and may serve to transport Ci and Smo to specific cellular compartments [126]. In the absence of Hh, the C-terminal domain of Ci is phosphorylated by PKA [130-133]. These modifications prime Ci for further phosphorylation by glycogen synthase kinase 3β (GSK3β) and various members of the CKI family of kinases [134-136]. These phosphorylations, in turn, promote binding of β-transducing-repeat-containing protein (βTrCP) to Ci [137]. βTrCP recruits the S phase associated protein kinase 1 (SKP1)-cullin 1 (CUL1)-F box protein (SCF) E3 ubiquitin ligase complex, which ubiquitinates Ci and targets it to the proteosome, leading to the selective degradation of its C-terminus [138]. This partial degradation frees the N-terminal repressor domain to enter the nucleus and repress gene transcription.

In the presence of Hh, activation of Smo promotes its association with HSC and activation of the kinase Fused (Fu). Fu phosphorylates Cos2 which allows the unprocessed Ci to dissociate from HSC and translocate into the nucleus [99, 129, 139-141]. The cytoplasmic protein Suppressor of Fu (SuFu) can restrain the translocation of Ci [142]. Recently, the structure of both *Drosophila* and human full length SUFU and the SUFU-GLI complex illustrated that SUFU exists as a dimer that clamps GLI in the middle [142]. Binding of GLI stabilizes a closed conformation of SUFU. Shh signaling, in contrast, favors the open SUFU conformation, promoting GLI dissociation and subsequent translocation of the activator forms of Ci/GLI into the nucleus. However, in *Drosophila*, SuFu loss of function has no effect on Hh signaling. In contrast, Cos2 deletion results in increased pathway activation, suggesting Cos2 functions as the main regulator of Ci [13]. Finally, in vertebrates, GLI1 is additionally regulated by novel kinases as well as other modifications such as acetylation and sumoylation [143-149].

While GLI2 and GLI3 are regulated in a similar manner as Ci, some divergence has occurred. The differences in regulation include the requirement of primary cilia for Hh signaling in vertebrates as well as the presence of additional regulators. In the absence of Shh, GLI2 and GLI3 transit through the primary cilia, a process that depends on the Cos2 ortholog KIF7 [150-154]. In addition, the kinases that regulate GLI processing – PKA, GSK3β, and CKI – are also localized to the basal bodies, and proteosomal degradation of GLI2 and GLI3 C-terminal transactivation domains occurs at basal bodies [104, 155-158]. Furthermore, GPR161, ACIII and other adenylyl cyclases required for cAMP generation, and hence PKA activation, localize to primary cilia in a TULP3 (Tubby-related protein 3) dependent manner [159-162]. In the presence of Shh, GLI2,

GLI3 and SUFU accumulate within the primary cilia. In vertebrates, in contrast to *Drosophila*, loss of SUFU leads to constitutive pathway activation, whereas loss of FU has no effect [163, 164].

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Non-canonical Hedgehog signaling and crosstalk with other pathways

Canonical Hh signaling mediated through the Ptch-Smo-Gli axis has been extensively studied in both development and cancer. In the last decade, however, an increasing number of studies have also uncovered the presence of non-canonical functions for Hh pathway components. These reports include 1) Ptch independent, 2) Ptch dependent, Smo independent, and 3) GLI independent functions of the Hh pathway. Furthermore, Hh signaling components can mediate cross-talk through direct interaction with other pathways. Finally, GLI can be activated through other pathways including K-RAS and TGFβ [165, 166]. These non-canonical signaling pathways regulate a variety of processes including apoptosis, cell cycle progression, axon migration, and cancer progression. Furthermore, there is evidence of integration between canonical and non-canonical signaling in certain contexts [167, 168].

Ptch independent functions of Hh

A Ptch independent function for Shh was identified in the development of the neural tube. In addition to its canonical role in differentiation of neural crest cells, Shh was found to inhibit the migration of these cells. Exogenous Shh seems to induce a

conformational switch in integrins and thereby reduce cell mobility. This was independent of Ptch signaling as mutant Shh proteins can signal through Ptch but have no effect on cell mobility. In fact, the N-terminal region of Shh required for influencing migration is distinct from the region that is required to bind Ptch [169].

Ptch dependent, Smo independent functions of Hh signaling

Recent studies have revealed a role for Ptch in apoptosis. The first report showed that Ptch induces apoptosis unless it is bound by Shh [170]. Because Ptch promotes apoptosis in the absence of ligand, it was proposed to act as a dependence receptor. Transient expression of Ptch, in the absence of Shh expression, resulted in increased apoptosis of 293T cells. Co-expression of Smo had no impact on cell death suggesting the canonical Shh pathway is not required. The authors also demonstrated that Ptch is cleaved at Asp1392, predominantly by caspase 3. Mutation of this residue inhibits the pro-apoptotic ability of Ptch [170]. Further studies showed that in the absence of Shh, Ptch interacts directly with DRAL (downregulated in rhabdomyosarcoma LIM-domain protein), recruits TUCAN (caspase recruitment (CAR)-domain containing proteins #8) or NALP1 (NLR family, pyr in domain containing 1) and activates caspase 9 to initiate apoptosis [171, 172]. Whether caspase 9 can also cleave Ptch and whether the recruitment of the DRAL/TUCAN/caspase 9 complex occurs before or after this cleavage remains to be determined [173].

The pro-apoptotic function of Ptch1 is also regulated by the E3 ubiquitin ligases Itch and, to a lesser extent, WWP2 [174]. Both Itch (Itchy E3 Ubiquitin Protein Ligase)

and WWP2 (WW Domain Containing E3 Ubiquitin Protein Ligase 2) physically interact with Ptch1 but not Ptch2. Ptch1 ubiquitylation on Lys1413 within the C-terminal domain promotes internalization and degradation of Ptch1. Reduced plasma membrane levels of Ptch1 inhibit Ptch1 pro-apoptotic activity and can lead to Gli1 transcriptional activation in the absence of Hh ligands [174]. Ptch mediated apoptosis has been observed in neuroepithelial and endothelial cells as well as in cancer cells under anchorage independent conditions [170].

Ptch1 can also regulate cell cycle progression. Endogenous Ptch was found to interact with phosphorylated, but not unphosphorylated, cyclin B1 in the absence of Shh [175]. This interaction prevents cyclin B1 from localizing to the nucleus [175]. The presence of Shh switches Ptch binding from cyclin B1 to GPRK2 in zebrafish. In the absence of GPRK2, Ptch remains bound to cyclin B1 [176]. In mice, conditional deletion of Ptch in interfollicular basal cells also results in increased nuclear localization of cyclin B1 [177]. Furthermore, $Ptch^{Q688X}$, a truncation mutant identified in patients with basal cell carcinoma, cannot bind cyclin B1 and does not inhibit G2/M progression [178].

Several studies suggest that in mammalian cells, the C-terminal domain of Ptch is not essential for canonical signaling [179, 180]. Insight comes from a spontaneous mouse mutant in which a 32bp deletion in the last exon generates $Ptch^{mes}$, a protein which lacks most of the C-terminal region. $Ptch^{mes}$ homozygous mice have normal spinal cord development compared to Ptch knockout mice which die around embryonic day 9 [58, 179]. In addition, $Ptch^{mes}$ mice have minimal alterations in Gli1 levels suggesting canonical Shh signaling is largely intact [180]. However, $Ptch^{mes}$ homozygous mice exhibit changes in body size, proliferation of basal cells, and mammary gland

development [180, 181]. Whether these reflect alterations in canonical or non-canonical functions of Ptch remains an open question.

Finally, two studies have reported novel functions for the C-terminal domain of Ptch. First, the C-terminal domain contains an SH3 interacting domain which mediates interaction with SH3 containing proteins including Grb2 and p85β [182]. Addition of recombinant ShhN results in increased Erk1/2 phosphorylation, even in the presence of Smo antagonists, once again suggesting non-canonical signaling [182]. However, whether the C-terminal domain of Ptch is required for Erk1/2 activation has yet to be determined. Second, expression of full length Ptch resulted in proteolytic processing to produce C-terminal fragments, which localize to the nucleus [183]. However, their biological function remains unknown.

Gli independent functions of Hh signaling

Gli independent functions of Shh signaling have been identified in dendritic spine formation, migration, neurotransmitter specification, and metabolism [173, 184-190]. Dendritic spine formation is regulated predominately through changes in the actin cytoskeleton. Addition of Shh alters spine formation whereas Gli depletion does not, suggesting a Gli1 independent function for Shh [184]. Moreover, treatment of NIH3T3 cells with recombinant Shh induces actin cytoskeletal rearrangements resulting in membrane ruffling even in the presence of transcriptional inhibition. It was shown that Smo can bind Tiam1, a Rac1 GEF. In fact, inhibition of the Tiam1-Rac1 pathway abrogates Shh mediated remodeling of the actin cytoskeleton [184].

Shh can induce tubulogenesis in 3D cultures of both human umbilical vein endothelial (HUVEC) and human cardiac microvascular endothelial (HMVEC) cells without Gli1 transcription. Treatment of endothelial cells with Hh ligands results in RhoA activation and stress fiber induction. This process is inhibited by Smo, G_i, and Rac1 antagonism suggesting a Smo dependent-Gli1 independent function for Hh in regulating actin cytoskeletal rearrangements [173]. A second study found that Smo-G_i coupling, but not Smo mediated Gli1 transcription, is required for fibroblast migration. In this system, Smo activation also requires PI3K (Phosphoinositide 3-kinase) signaling for activation of RhoA and Rac1 [185].

Commissural axons turn towards a Shh gradient, and inhibition of transcription does not reduce axon guidance, suggesting a Gli independent function. Instead, Shh signaling through Smo stimulates activation of the Src family kinases, Src and Fyn, which are required for growth cone turning [186]. Additionally, Smo-mediated Shh signaling regulates lamellipodia formation and migration. This process requires the activity of 5-lipoxygenase, an enzyme that generates leukotrine precursor from arachidonic acid [187]. Smo dependent Shh mediated neurite outgrowth in motor neurons requires a functional 5-lipoxygenase pathway [188].

Another example of non-canonical Smo activity is found in the developing brain where Ca^{2+} spike activity regulates neurotransmitter specification. Addition of recombinant Shh results in increased Ca^{2+} spike activity. Smo inhibition reduces this and Smo coupling to G_i is required for inducing Ca^{2+} spike acidity in response to Shh. The recruitment of G_i proteins leads to increased concentrations of inositol triphosphate and Ca^{2+} release [189].

Smo mediated induction of Ca^{2+} flux also plays a role in modulating metabolism. Smo activation, through recombinant Shh or SAG, promotes phosphorylation of AMP-activated protein kinase (AMPK) and pyruvate kinase M1/M2 (PKM2), induces opening of Ca^{2+} channels, and results in rewiring towards glycolysis in adipocytes [190].

Hedgehog ligand maturation, secretion, and transport

All hedgehog proteins are synthesized as precursor proteins that undergo a series of post-translational modifications which regulate secretion, signaling range, and signal potency. The N-terminus contains a signal sequence which is cleaved upon entry into the secretory pathway [2]. The C-terminal domain catalyzes an autoprocessing event to generate a ~25kDa C-terminal fragment and a ~19kDa N-terminal fragment (ShhN) that retains all signaling activity [14, 15, 191, 192]. ShhN is further modified and secreted from cells while the C-terminal domain is degraded by endoplasmic reticulum associated degradation [193]. Alternatively, the C-terminal domain may also be secreted from cells, although the significance of this remains to be determined [194].

Autoprocessing is required for optimal signaling activity [14, 192]. In *Drosophila*, unprocessed forms of Hh are able to signal albeit not as effectively as processed Hh [14, 192]. Data from human embryonic kidney cells suggests that full length, unprocessed Shh can signal in a short range, direct cell-to-cell context [195]. Autoprocessing occurs between conserved, adjacent glycine and cysteine residues. During autoprocessing, the peptide bond between these residues is rearranged to form a thioester. Subsequently, a

cholesterol moiety attacks the carbonyl of the thioester and becomes covalently attached to the C-terminus of ShhN [192]. *In vitro* studies indicate that the 3β hydroxyl group of cholesterol acts as the nucleophile in this reaction and other sterols containing a 3β hydroxyl group can substitute for cholesterol [91]. However, only cholesterol modified Hh ligands have been identified. The cholesterol modification is not required for Shh binding to Ptch. Moreover, over-expression of a cholesterol unmodified form of Shh is active in patterning of the neural tube. The main function of the cholesterol moiety is in regulating the spatial distribution of ShhN [16, 196-199].

The N-terminus of ShhN is modified by covalent attachment of the 16-carbon fatty acid palmitate to the N-terminal cysteine [18, 19]. Shh palmitoylation is catalyzed by Hedgehog acyltransferase (Hhat), a multipass transmembrane enzyme that belongs to the membrane bound O-acyltransferase (MBOAT) family [19]. Palmitoylation is thought to occur through a thioester linkage to the cysteine residue, which then rearranges via an intramolecular S-to - N shift to produce an N-linked palmitate [18, 200]. A thioester intermediate has yet to be isolated [19]. Palmitoylation requires the presence of the signal sequence but is independent of the autoprocessing event and the cholesterol modification [19]. Multiple studies have established that palmitoylation of Shh by Hhat is critical for signaling activity [17, 20-23]. Recently, ShhN was found to be modified with fatty acids other than palmitate, such as palmitoleoyl (C16:1), stearoleyl (C18:1), myristolel (C14:1), oleoyl (18:0), and myristoleyl (C14:0) [201]. Modification with unsaturated fatty acids resulted in reduced localization to lipid rafts and therefore reduced signaling, suggesting that differential fatty acylation of Shh may serve as a novel mode of signaling regulation. Importantly, Hhat can transfer fatty acids of various lengths onto Shh [19].

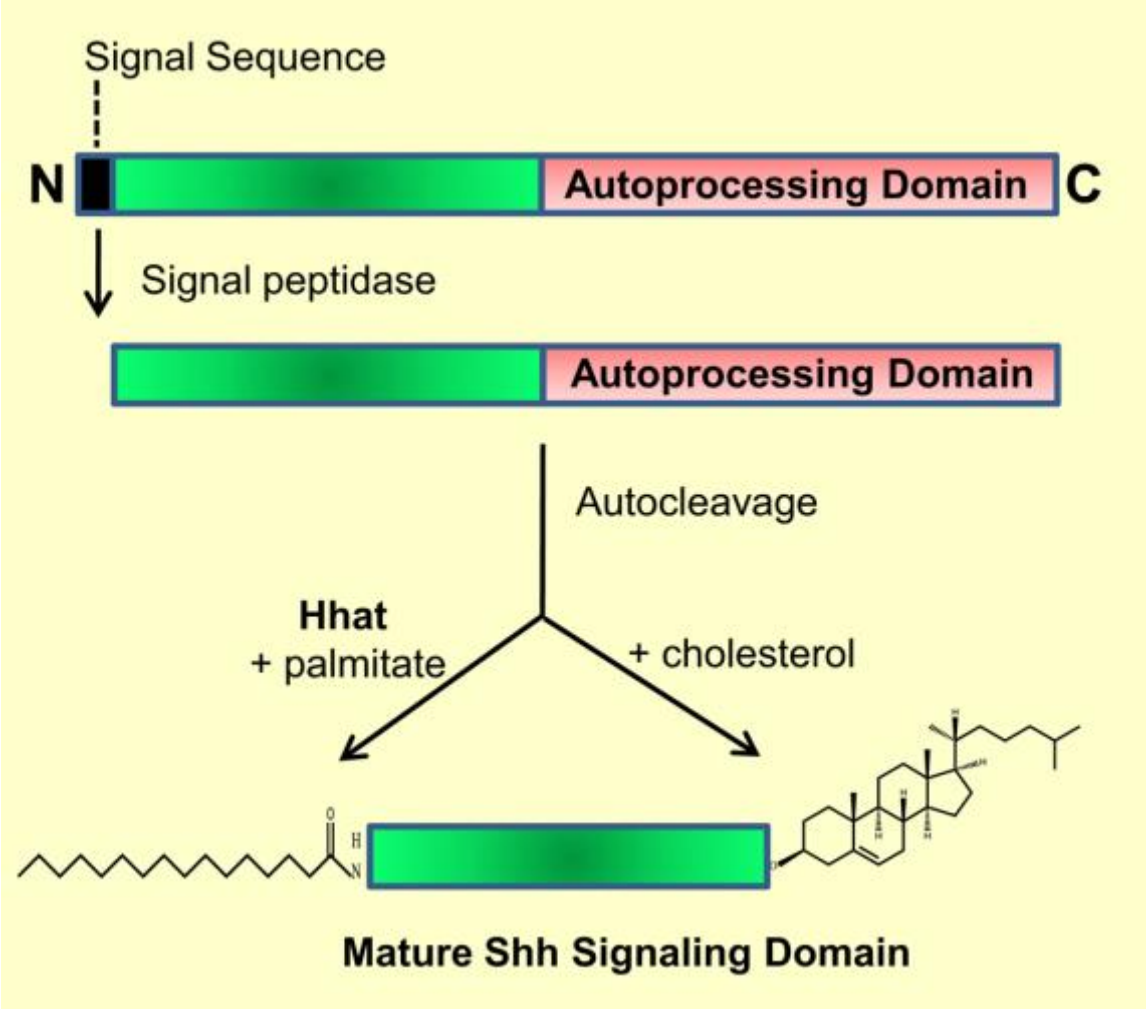

Figure 1.2 Generation of the mature Shh signaling domain. The full length precursor Shh protein contains an N-terminal signal sequence, which is cleaved upon entry into the secretory pathway. The C-terminal domain of Shh catalyzes an autoprocessing event to generate the ~19kDa signaling domain. During autoprocessing, cholesterol is covalently attached to the C-terminus of the signaling domain. In a separate reaction, Hhat catalyzes the attachment of a palmitate moiety onto the N-terminal cysteine residue of the Shh signaling domain. Reprinted from Trends in Molecular Medicine, 18(4), Marilyn D. Resh, Targeting protein lipidation in disease, 206-14, Copyright 2012 with permission from Elsevier.

The cholesterol and palmitic acid modifications confer to Hh an affinity for cellular membranes [202]. Nevertheless, Hh can signal over a long range including up to 300μm in the limb bud of vertebrates [13]. In most tissues, Hh ligands are secreted from polarized cells and both apical and basolateral pools of ligand have been detected [203-205]. Studies in the *Drosophila* wing disc suggest that Hh released from the apical surface is first internalized and then released at the basolateral surface. This interpretation is supported by experiments showing that blocking endocytosis in Hh producing cells leads to Hh accumulation [203]. The apical plasma membrane is enriched for cholesterol and glycosphingolipids, which may facilitate anchoring of the dual lipid modified Hh ligand [206]. Cholesterol and triglycerides are known to undergo apical to basal transcytosis [207]. Therefore, it is possible that the lipid modified Hh is transported in a similar fashion. The fact that lipid unmodified Hh does not localize to basolateral surfaces is consistent with these findings [203].

The release of the dual lipid modified Hh ligands is mediated by the twelve-pass transmembrane protein Dispatched (Disp) and the secreted protein Scube2 [202, 208-212]. Two Disp homologs have been identified in mice – DispA and DispB. DispA is highly expressed and is required for all Hh signaling during early embryogenesis. The contributions of DispB have yet to be determined [213]. Cells deficient in Disp fail to secrete cholesterol modified Hh ligands resulting in ligand accumulation within cells [209, 213-216]. Hh constructs that cannot be modified with cholesterol bypass the requirement for Disp mediated release [211, 217]. Disp, like Ptch, is a member of the bacterial RND family of proteins and contains an SSD domain, which mediates the interaction with the cholesterol moiety of Hh [209, 211, 217]. In addition to its role in Hh

secretion, two studies reveal a role for Disp in facilitating intracellular transport of Hh from the apical to the basolateral surface. First, Disp was found to colocalize with Hh in vesicles and multivesicular bodies [203]. Second, Hh endocytosis at the apical surface is abrogated in Disp mutant wing discs and results in decreased long range signaling [218].

The requirement for Scube2 in mediating long range Shh signaling was first identified in zebrafish [210, 219, 220] and later verified using cell culture models [211, 212]. Scube2 is part of the Scube family of secreted proteins, which also includes Scube1 and Scube3. Simultaneous knockdown of all three Scube proteins phenocopies loss of Hh mutants [221]. Scube2 interacts with a part of the cholesterol moiety that is distinct from the site of Disp interaction [211]. Scube2 and Disp act synergistically to release lipid modified Shh from cells [211]. Scube2 is only found in vertebrates and it is still unclear whether invertebrates have a Scube2 like protein or whether Hh is released via other mechanisms.

Recent studies suggest that dually lipidated Shh is released from cells by proteolytic processing or shedding [222-224]. ShhNp shedding is increased when Shh is co-expressed with disintegrin and metalloprotease (ADAM) family member 17 whereas inhibition or knockdown of ADAM17 reduces ShhNp shedding [224]. Structural analysis demonstrated that the N-terminal peptide obstructs the zinc coordination sites within ShhNp required for binding to Ptch [222]. Palmitoylation is required for sheddase mediated release of Shh as nonpalmitoylated ShhC25S exhibits defective shedding [222]. Processed Shh has also been detected in mouse brain samples [223]. Further studies are needed to delineate the effects of sheddase activity on Shh signaling. One report suggests

that in the absence of ADAM17, accumulation of cell tethered Shh leads to increased range of signaling in pancreatic cancer cells [225].

Various mechanisms have been proposed for the transport of Hh ligands through tissues including secreted multimeric complexes, lipoprotein containing particles, filopodia-like structures, and exosomes [226]. Shh can be recovered from tissue culture cell supernatants in monomeric forms as well as high molecular weight multimeric complexes [21, 227-229]. Multimerization is dependent on the presence of both lipid modifications [21, 198, 206, 228]. Both modifications promote association of ShhN with sterol-rich membrane microdomains, which may be necessary for multimerization [21]. Importantly, the multimer complexes show increased signaling potency both *in vitro* and *in vivo*. The mechanism of multimerization remains unknown.

Lipoprotein particles have been proposed to serve as carriers for Hh ligands [230, 231]. Lipoproteins form a phospholipid monolayer that surrounds a center of triglyceride and esterified cholesterol. In *Drosophila*, there is evidence implicating lipoprotein particles in long range Hh signaling. Knockdown of lipophorin, the insect lipoprotein, in imaginal discs reduces the range of Hh signaling. Interestingly, the signaling range of Wingless was also reduced [206, 231]. Wingless and the related Wnt proteins are secreted, fatty acylated molecules. Wingless co-purifies with lipophorins and requires lipophorin expression for proper signaling. A recent study reported that circulating lipoprotein-associated Hh modulates *Drosophila* larval growth by coupling nutrient availability and development [232]. In humans, analysis of plasma lipoproteins identified an association of small amounts of Ihh, but not Shh or Dhh, with very low density lipoproteins [233]. These Ihh containing lipoproteins increased the survival of endothelial

cells. Additionally, lipoproteins may also play a role in the reception of the Hh signal. For instance, Ptch was shown to interact with and internalize lipophorins [234]. Furthermore, in the developing forebrain, Shh signaling requires the low density lipoprotein receptor related protein (LRP) 2 [235]. LRP2 mutations in humans or deletion in mice reduces Shh signaling and leads to holoprosencephaly, a hallmark of defective Shh signaling during development [236, 237]. In this respect, the Hh pathway is once again similar to the Wnt pathway as Wnt proteins signal by binding to the receptor Frizzled and co-receptor LRP5/6 [238].

Several studies have observed a role for long, dynamic filopodia-like structures called cytonemes in Shh signaling. Cytonemes were first observed extending from receptor cells to contact Decapentaplegic (Dpp) producing cells in *Drosophila* [239]. The discovery of various receptors on these structures suggested that it may be a general mechanism for directed cell-to-cell communication [239, 240]. In the *Drosophila* ovarian niche, Hh is produced by cap cells and localizes to cytonemes emanating from these cells [241]. Blocking Hh signal transduction in adjoining cells induced the formation of longer Hh coated cytonemes from producing cells, resulting in increased range of signaling [241]. In addition to Hh itself, various Hh signaling components also localize to cytonemes [203, 204]. Similar actin based filopodia-like structures were also observed in the wing discs of *Drosophila* and in chick embryos [203, 205, 242, 243]. In chick embryos, Shh producing cells extend filopodia containing ligand and target cells extend filopodia containing receptors [243]. Membrane to membrane contacts formed by these specialized structures ensures a controlled method of communication.

Another potential mode of transport for Hh proteins are argosomes or exovesicles, extracellular vesicles containing a lipid core [244]. In the developing mouse embryo, Shh signaling is required for left-right symmetry establishment [31, 245]. During symmetry breaking in the mammalian ventral node, exovesicles were observed carrying Shh, among other molecules, to target cells [246]. Recent reports suggest that Hh loaded exovesicles are released from cytonemes and that the endosomal sorting complex required for transport (ESCRT) is required for this process [247, 248]. Indeed, knockdown of ESCRT genes in *Drosophila* wing imaginal discs inhibits long range Hh signaling [248]. In *C. elegans*, mutations in V0-Atpase, the enzyme required for exosome secretion, result in defective release of Hh related peptides [249]. These different modes of Hh transport highlight the numerous functions of Hh. Whether similar or distinct mechanisms of Hh transport are present in different tissues or whether the mechanism determines the range of Hh transport remains an open question.

Hedgehog acyltransferase

Protein palmitoylation

Hundreds of cytoplasmic and membrane proteins are modified with lipids [250-252]. The three common types of lipid modifications are fatty acids, isoprenoids, and GPI anchors. Protein lipidation can modulate protein trafficking, localization, structure, and function. In addition, lipidation increases association with lipid rafts, membrane microdomains enriched in cholesterol, glycosphingolipids, and signaling molecules. Given the wide range of proteins subjected to this type of post translational modification,

it is not surprising that lipidation plays an integral role in various cellular processes including proliferation, motility, neuronal excitation, and immune response [250-254].

Palmitoylation refers to the attachment of a 16-carbon saturated fatty acid onto a target lipid or protein. However, the term may also be applied to acylation with longer chain fatty acids as many proteins incorporate fatty acids of various lengths [19, 255-257]. Two families of palmitoyl acyltransferases catalyze the transfer of palmitate onto target proteins: the Asp-His-His-Cys - cysteine rich domain (DHHC-CRD) family and the membrane bound O-acyltransferase (MBOAT) family [250]. These enzymes transfer palmitate from palmitoyl-CoA onto their substrates through either S-palmitoylation or, less commonly, through N-palmitoylation. S-palmitoylation refers to the attachment of palmitate onto an internal cysteine residue via a thioester linkage [250, 252]. Because of the labile nature of thioester bonds, modified proteins can be subjected to rounds of palmitoylation and depalmitoylation. This allows for dynamic regulation of protein localization and function. In contrast, N-palmitoylation is a stable modification in which palmitate is attached to an N-terminal cysteine through an amide bond. To date, only a handful of proteins are known to undergo N-palmitoylation: the $G\alpha_s$ subunit, the Hh family of molecules, and the *Drosophila* EGFR-like ligand Spitz [19, 258-260].

Palmitoylated proteins include Ras GTPases, ion channels, GPCRs, immune cell receptors, and Src family kinases (SFKs) [250]. Ras GTPases regulate cell proliferation, differentiation, and motility. The three major Ras isoforms H-Ras, N-Ras, and K-Ras are first prenylated in the cytosol. Subsequently, H-Ras and N-Ras are palmitoylated in the Golgi, whereas K-Ras contains a polybasic amino acid cluster which facilitates targeting to the membrane [250]. H-Ras and N-Ras undergo cycles of palmitoylation and

depalmitoylation, which is essential for proper localization of both proteins within plasma and Golgi membranes [261-263]. Downstream signaling depends on the subcellular localization of Ras proteins, highlighting the importance of dynamic palmitoylation [264].

Palmitoylation of voltage gated Ca^{2+} channels regulates localization and ion sensing activity [265, 266]. Moreover, the channel interacting protein kChIP2 is palmitoylated, which localizes it and its associated kv4.3 voltage gated potassium channel to the plasma membrane [267].

GPCRs are seven transmembrane receptors that are activated by diverse stimuli, including hormones, lipids, and ions [250]. Palmitoylation of various GPCRs is required for proper folding, targeting to the cell membrane, and therefore coupling to G proteins [268, 269]. GPCR signaling is further regulated by fatty acylation of the α and βγ subunits of G proteins, which is required for proper targeting to the plasma membrane [260].

In the immune system, palmitoylation is required for CD4 and CD8 localization into lipid rafts and subsequent signaling [270-273]. Furthermore, palmitoylation regulates the activation of the Src family kinases Lck and Fyn, which are activated upon antigen recognition by the T cell receptor. All SFKs are modified with the 14-carbon fatty acid myristate at the N-terminus. In addition to myristate, a second hydrophobic signal – a polybasic cluster of amino acids or a palmitate moiety – is required to target SFKs to lipid rafts [250, 274, 275]. Raft association is essential for Lck and Fyn activity after T cell receptor activation. Fatty acylation also regulates the localization and function of several signaling proteins required for effective B cell activation [254].

Membrane bound O-acyltransferases

The MBOAT family of enzymes was initially identified through global sequence alignment [276]. MBOAT enzymes are characterized by an invariant His residue surrounded by hydrophobic residues and a highly conserved Asp/Asn residue surrounded by moderately hydrophobic residues. The highly conserved His or Asp/Asn residues are thought to be involved in catalysis as mutation of these residues, separately or together, reduces or abolishes catalytic activity [277-280]. MBOAT proteins are predicted to contain between 8-13 transmembrane domains (TMDs). To date, the crystal structure has not been solved for any MBOAT enzyme. Structural information comes from mutational analyses and topological determination [281, 282].

Mammalian MBOAT proteins can be divided into three subfamilies based on sequence homology or substrate preference [258]. First, the ACAT (acyl:CoA cholesterol-acyltransferase) family includes ACAT1, ACAT2, DGAT1 and DGAT2 (acyl:CoA diacylglycerol-acyltransferase). ACAT1 and ACAT2 mediate cholesterol ester formation in mammalian cells. DGAT1 catalyzes the formation of triacylglycerol from diacyglycerol and long chain fatty acid CoAs. Second, the LPLAT (lysophospholipid acyltransferase) family includes MBOAT1, MBOAT2, MBOAT5, and MBOAT7. These enzymes catalyze the reacylation of multiple classes of lysphospholipids at the sn-2 position [283]. Finally, the PAT (protein acyltransferase) subfamily of enzymes transfers fatty acids onto protein substrates and consists of Hhat, Porcupine, ghrelin O-acyltransferase, and the mammalian homologue of glycerol uptake protein 1. The function of Hhat is discussed in detail in the following section.

Porcupine (Porcn) is the acyltransferase for the Wnt family of secreted signaling molecules [284-290]. Wnts play an important role in diverse processes during embryogenesis as well as adult tissue homeostasis [238, 291]. Porcn catalyzes the attachment of palmitoleate, a 16-carbon monounsaturated fatty acid, onto a conserved serine residue in Wnts [289, 290]. This modification is required for proper trafficking and secretion of Wnts. Mutation of the serine residue to an alanine results in accumulation of Wnt proteins in the endoplasmic reticulum [288]. In addition, Ser to Ala mutants cannot interact with Wntless, a membrane protein required for Wnt secretion [292, 293]. Furthermore, the structure of Wnt in complex with its receptor Frizzled clearly demonstrates that the lipid modification is directly involved in binding Frizzled [294, 295].

Porcn is essential for Wnt activity during development, and mutations in the PORCN gene result in focal dermal hypoplasia, an X-linked developmental disorder [296-298]. Wnt signaling is also upregulated in a number of cancers including breast, colon, and head and neck [299-301]. Therefore, Porcn is an attractive target for downregulating Wnt signaling in these tumors. In fact, a small molecule Porcn inhibitor, LGK974, is currently in clinical trials for Wnt driven tumors [302].

Ghrelin O-acyltransferase (GOAT) catalyzes the attachment of octanoic acid onto ghrelin, a small peptide appetite stimulating hormone [279, 303-306]. Ghrelin is produced in the gut and is required for regulating food intake and energy homeostasis [307]. The active, mature form of ghrelin is produced after cleavage and acylation and acylation is required for all known function [306]. Mutation of the conserved His or Asn residues in GOAT abolishes catalytic activity [279]. GOAT can use fatty acids up to

tetradecanoic acid; however, only octanoylated ghrelin has been identified *in vivo* [306]. GOAT knockout mice do not have acylated ghrelin in their circulation. Consequently, when placed on a high fat diet, they have reduced weight gain and fat mass compared to wildtype mice. GOAT is expressed in endocrine cells of the stomach as well as in the pituitary gland, whereas ghrelin is not expressed in the latter, suggesting GOAT may acylate additional substrates [307, 308].

The mammalian homolog of glycerol uptake protein 1 (GUP1) is also known as Hhat-like (Hhatl) due to a high degree of homology with Hhat. In *Saccharamyces cerevisiae*, GUP1 is required for glycerol import [309]. On a molecular level, GUP1 regulates the synthesis of C26:0-containing diacylglycerol anchors of mature GPI-anchored proteins [277]. Inhibition of GUP1 leads to defects in cell wall composition, glycerol uptake, and reduced growth under various environmental stresses [309, 310]. In mammalian cells, one report suggests that GUP1 interacts with both Shh and Hhat and interferes with Shh palmitoylation [311]. However, Shh palmitoylation was monitored indirectly and in the absence of co-expression with Hhat. We have previously shown that little to no palmitoylation occurs when Shh is overexpressed in the absence of Hhat expression. Therefore, further studies are necessary to assess the potential role of GUP1 on Shh palmitoylation.

Hedgehog acyltransferase

Four groups independently identified Hhat as a critical component of the Hh signaling pathway [20, 22, 23, 208]. In *Drosophila*, the Hhat orthologs are named Raspberry (Rasp), Sightless, Central Missing, and Skinny Hedgehog and will be referred

to as Rasp for clarity. Rasp mutants die at the pupal stage and maternal loss of Rasp causes embryonic lethality [20]. Rasp mutants phenocopy defects of Hh mutants in segment polarity, photoreceptor differentiation, and wing disc development [22, 23, 208]. In the developing eye, photoreceptor differentiation is regulated by Hh signaling, and Rasp mutants show dramatic decreases in Hh target gene expression [22, 208]. Importantly, overexpression of Hh in Rasp mutants is not sufficient to activate target gene transcription [20]. Rasp mutants, however, have normal Hh protein expression, autoprocessing, cholesterol incorporation, and secretion suggesting that Rasp regulates Hh signaling activity through another mechanism. Insight came from the observation that the sequence of Rasp shared homology with the MBOAT family of enzymes suggesting that Rasp functions to lipidate Hh [20, 22, 23, 208].

The role of Hhat in Hh signaling is conserved in mice. Hhat knockout mice die shortly after birth and recapitulate many defects exhibited by Shh or Ihh knockout mice [21, 312]. Defects in neural development and limb patterning reflect deficiencies in both short and long range Shh signaling, highlighting the importance of palmitoylation on Shh activity. In addition, Hhat knockout mice have dwarfed limbs due to severely reduced chondrocyte proliferation, resembling the phenotype of Ihh knockout mice [21].

Biochemical studies established that Hhat is, in fact, the palmitoyl acyltransferase for the Hh family of secreted molecules [19, 313]. Hhat is a ~50kDa multipass transmembrane protein that localizes to the endoplasmic reticulum and Golgi, where it colocalizes with Shh [19]. *In vitro* and cell based studies demonstrated that radiolabeled palmitate is incorporated onto wild type Shh only in the presence of Hhat. In cells, the N-terminal signal sequence is required for palmitoylation, presumably because it facilitates

entry into the secretory pathway where Hhat resides. Removal of the signal sequence exposes an N-terminal cysteine (ShhC24) which is palmitoylated. Mutation of this cysteine residue to an alanine (ShhC24A) abolishes palmitoylation [19, 314]. Interestingly, a serine in this position (ShhC24S) can serve as a substrate, although with less efficiency, for human Hhat [314]. In contrast, the *Drosophila* Cys to Ser mutant cannot be palmitoylated by Rasp [314]. These findings explain why HhC24S mutant *Drosophila* show no Hh activity whereas ShhC24S mice exhibit low levels of Shh signaling and milder phenotypes than Shh knockouts [21, 315]. Finally, Shh mutant proteins that cannot undergo autoprocessing, and therefore do not incorporate cholesterol, are still efficiently palmitoylated by Hhat [19].

In *Drosophila*, Rasp is also the palmitoyl acyltransferase for Spitz [259]. Spitz is one of three EGFR-like ligands in flies, which also include Keren and Gurken. Keren, which often functions redundantly with Spitz, mediates various processes including photoreceptor development, border cell migration, and intestinal stem cell maintenance [316-318]. Gurken mediates the establishment of the embryonic axis [319]. Activation of Hh signaling fails to rescue photoreceptor differentiation in Rasp mutants suggesting the presence of an additional substrate for Rasp. The authors noted that Spitz target genes were down-regulated in Rasp mutants. Further analysis showed that cleavage of the signal sequence of Spitz exposes a Cys residue which is palmitoylated by Rasp. This modification restricts the spatial distribution of Spitz within tissues. The sequence following the palmitoylated Cys residue in Spitz shows little homology to that of Hh; however, both proteins contain several basic amino acids. Gurken and Keren may also

serve as substrates for Rasp because both have Cys residues immediately following the signal peptide as well as basic amino acids in their N-terminal regions [259].

The presence of additional substrates for Rasp suggests that Hhat may also have additional substrates. *In vitro* studies demonstrated that the first six amino acids of Shh (CGPGRG) are sufficient for recognition and palmitoylation by Hhat [314]. Ihh and Dhh are presumed to be substrates for Hhat based on sequence homology. In fact, the first six amino acids following the signal peptide are conserved in all three mammalian Hh ligands and direct evidence of Dhh palmitoylation by Hhat was recently provided [313]. The influence of each amino acid on palmitoylation was also examined [314]. A Cys residue is required at the N-terminus, although a Ser residue can also incorporate palmitate to a lesser extent. In addition, a positively charged residue is required within the first 7 amino acid residues, and a Lys in the second position is not tolerated. These results were used in conjunction with a bioinformatics screen to identify secreted proteins which have an N-terminal Cys residue after signal sequence cleavage. While a number of proteins fit this criteria, additional substrates have yet to be validated for mammalian Hhat [314].

Hedgehog signaling in cancer development and maintenance

Hh signaling is required during embryogenesis but is downregulated in adult tissues, with the exception of stem cell compartments. Aberrant reactivation of the pathway is associated with initiation and progression of numerous tumors [11]. Upregulation of Hh signaling occurs either through mutations in various pathway

components (ligand independent activation) or through upregulation of Shh expression in the absence of mutations (ligand dependent activation). The mechanism of aberrant Hh signaling dictates the design of appropriate treatment options.

Ligand independent activation of Hh signaling

The link between Hh signaling and cancer was first established with the identification of PTCH1 mutations in patients with Gorlin syndrome [320-324]. Affected individuals have high incidence rates of basal cell carcinomas (BCCs), medulloblastomas (MBs) and rhabdomyosarcomas. $Ptch1^{+/-}$ mice develop BCC like lesions upon deletion of the wild type allele or upon exposure to ultraviolet radiation [325, 326]. Moreover, skin specific deletion of Ptch1 leads to the formation of BCC lesions [327, 328]. Furthermore, alterations in other pathway components, such as constitutive activation of Smo, heterozygous loss of SuFu, or GLI1/2 overexpression, specifically in the skin also lead to BCC development [325, 326, 329-331]. In transgenic mice which overexpress GLI in the epidermis, the strength of the Hh pathway activation correlates with the type and aggressiveness of the tumor [330].

Medulloblastoma is the most commonly diagnosed brain tumor in children and arises from over-proliferating granule neuron progenitors/precursors (GNPs). During development, GNP proliferation is restricted by Ptch1 until exposure to Shh [332]. About a quarter of MB patients have mutations in PTCH1 and SUFU [333-335]. Inactivation of one Ptch allele leads to MB development in 15-43% of mice [58, 336] and exposure to ionizing radiation increases the incidence rate to 80% [337, 338]. Additionally, miRNAs

targeting SMO and GLI1 are down-regulated in human MB [339]. Activation of Hh signaling in the GNP lineage using an inducible *Math1*-CreER transgene leads to MB development in all mice [340, 341]. Taken together, these studies demonstrate that mutations leading to Hh pathway activation promote the development of BCC and MB.

Ligand dependent activation of Hh signaling

Increased expression of Shh ligands either by tumor or stromal cells has also been observed in various tumors. It is becoming clear that tumor derived Shh can signal in both an autocrine and paracrine fashion in these tumors. Shh secreted by tumor cells can initiate signaling within tumor cells to promote survival or proliferation. Alternatively, it can initiate signaling in stromal cells to create a protumorigenic microenvironment. In other settings, Shh secreted by stromal cells can activate Shh signaling in tumor cells.

Ligand dependent Shh signaling has been reported in pancreatic, upper gastrointestinal, breast, ovarian, prostate, colorectal, and lung cancers [342-351]. Inhibition of Hh signaling using the ligand neutralizing 5E1 antibody, SMO or GLI1 knockdown, or Smo inhibitors reduces proliferation of tumor cells both *in vitro* and *in vivo* suggesting autocrine signaling promotes tumor growth [342-350]. Numerous studies also suggest that paracrine signaling is important for the progression of certain tumors. Shh overexpression in the prostate cancer cell line LNCaP does not alter proliferation *in vitro* but produces faster growing tumors *in vivo* [352]. In these tumors, Shh target gene expression (Gli1, Gli2, and Ptch1) is upregulated in the stroma but not in the tumor. Similarly, analysis of tumor tissues from pancreatic, prostate, ovarian, and colorectal

tumors demonstrated increased activation of Hh signaling in the stroma. The contribution of autocrine and paracrine Hh signaling to tumor progression may be tissue or context dependent.

Finally, there is evidence that certain tumors can respond to Shh secreted by stromal cells. For example, B cell lymphoma cells from the *Eμ-Myc* model require co-culture with stromal cells. Treating co-cultures with cyclopamine or 5E1 induces apoptosis of lymphoma cells. Alternatively, treatment of lymphoma cells with recombinant Shh or Ihh is sufficient to promote survival in the absence of stromal cells. Non-Hodgkins lymphoma and myeloma cells from patients also show reduced survival in the presence of cyclopamine [353]. Stromal Shh has also been identified in gliomas. In a mouse model of platelet-derived growth factor-induced gliomas, the expression of both Shh and Gli1 is upregulated and correlates with tumor grade [354]. In this model, astrocytes and endothelial cells in the perivascular niche promote tumor progression by producing Shh. Moreover, Shh producing astrocytes and endothelial cells are also found in human glioblastoma multiforme [354].

Hh signaling as a restraint on tumor growth

In pancreatic cancer, promising preclinical data showing that inhibition of Shh signaling reduces tumor growth ended with rather disappointing results in clinical trials, which showed only a slight increase or even a decrease in overall survival depending on the study [355]. Further analysis using either genetic inactivation of Shh or chronic saridegib treatment in genetically engineered mouse models identified a potential role for

Shh in restraining pancreatic cancer progression. Rhim and colleagues found that Shh deletion in Pdx1-Cre;$Kras^{LSL\text{-}G12D/+}$;$p53^{fl/+}$;$Rosa26^{LSL\text{-}YFP/+}$(PKCY) mice leads to earlier tumor development and decreased survival [356]. In addition, the tumors are characterized by undifferentiated or poorly differentiated histology. Furthermore, when comparing human tumors of different histologies, SHH and GLI-1 expression are reduced in undifferentiated tumors compared to tumors of other histologies. Shh deletion resulted in tumors which lack the characteristic desmoplasia associated with pancreatic cancer. Instead, the tumors have increased number of blood vessels, which correlates with decreased autophagy and increased proliferation. Similar results were obtained when PKCY mice were treated with saridegib, a Smo inhibitor, for longer periods of time. Notably, increased vasculature in Shh deleted tumors makes them more responsive to VEGF inhibition, and treatment with a VEGFR2 antibody significantly improves survival. Importantly, SHH PKCY mice had a 10-fold increase in Ihh expression. Ihh expression was not analyzed in tumors treated with saridegib or in human undifferentiated tumors. It will be important to understand whether Ihh can compensate for Shh loss.

In another study, genetic deletion of Shh in a different mouse model of PDAC also leads to increased formation of high grade lesions, increased microvasculature, and reduced survival [355]. Furthermore, inhibition of Hh signaling with vismodegib leads to decreased numbers of GLI-1 expressing stromal cells whereas pathway activation with a SMO agonist increases the number of myofibroblast-like cells. Therefore, Hh signaling is required for the formation of the stromal desmoplasia associated with pancreatic cancer.

The authors suggested that Hh inhibitors, which reduce desmoplasia and increase tumor vasculature, may be successful when combined with more potent chemotherapeutics.

Finally, Ozdemir and colleagues reported that depletion of myofibroblasts in another model of pancreatic cancer leads to the formation of more undifferentiated tumors and decreases survival [357]. In addition, these tumors show decreased effector T-cell and increased regulatory T-cell infiltration, which was associated with increased CTLA-4 expression. Treatment with anti-CTLA-4 antibodies extends overall survival of myofibroblast depleted tumor bearing mice compared to control tumor bearing mice. In the context of pancreatic cancer, both myofibroblast depletion and Shh inhibition lead to the development of more undifferentiated tumors. Therefore, it will be interesting to assess the combination of Shh inhibition with anti-CTLA4 antibodies on pancreatic cancer progression.

Hh signaling in cancer stem cells

The cancer stem cell (CSC) hypothesis postulates that a rare population of tumor cells can initiate and sustain tumor formation. These cells are similar to healthy stem cells in their ability to self renew and differentiate; however, CSCs do not necessarily arise from the malignant transformation of stem cells [358]. Developmental pathways, such as Hh, Wnt, Notch, and TGFβ, regulate self renewal of normal stem cells and are often upregulated in CSCs [359, 360]. CSCs have been identified in various tumors including hematopoietic, pancreatic, brain, prostate and breast neoplasms [361-365]. The hematopoietic system has a hierarchical organization with clearly defined stem cell and progenitor populations, and CSCs have been identified in hematopoietic malignancies

such as chronic myeloid leukemia and multiple myeloma [363]. In chronic myeloid leukemia, Bcr-Abl expressing leukemic stem cells activate Hh signaling through Smo upregulation. Inhibition of Smo with cyclopamine reduces colony formation *in vitro* [366]. Expression of a constitutively active Smo accelerates disease progression whereas loss of Smo reduces stem cell numbers and inhibits retransplantability of disease [366, 367]. In multiple myeloma, CSCs exhibit enhanced Hh signaling. Inhibition of Hh signaling with cyclopamine reduces the CSC compartment, possibly by inducing terminal differentiation [368].

Within the last decade, CSCs have also been identified in a number of solid tumors. In pancreatic cancer, CSCs (characterized by $CD44^+CD24^+ESA^+$ staining) show increased Hh expression and an enhanced ability to initiate tumor formation when injected into immunocompromised mice in limiting dilutions [361]. In addition, treatment of pancreatic cancer cells with a Smo inhibitor reduces the percentage of CSCs in the primary tumor and decreases metastasis formation [367]. In gliomas, Hh signaling regulates the self renewal of $CD133^+$ glioma CSCs. Smo knockdown or cyclopamine treatment reduces tumor volume *in vivo* [362]. Furthermore, cyclopamine treatment abrogates the ability of glioma CSCs to initiate tumors in mice [369]. In prostate cancer, the CSC population lacks differentiation markers, is resistant to Doxetacel treatment, and shows increased tumorigenic capacity *in vivo*. These cells also have increased Hh and Notch signaling, and inhibition of both pathways depletes these cells and helps reduce chemoresistance [365]. The role of Hh signaling has also been evaluated in breast CSCs, which will be discussed in a later section.

Hedgehog signaling in mammary gland development and cancer

Hh signaling in mammary gland development

Mammary gland development can be divided into three stages: embryonic, non-parous, and pregnant/lactating [351, 370]. The mammary gland consists of epithelial and stromal cells. Epithelial cells form ducts and milk-producing alveolar cells. The luminal, milk producing cells are embedded in a system of basal, myoepithelial cells, and rudimental ductal trees form during embryonic development. Ductal outgrowth and branching is expedited during puberty in response to estrogen and progesterone. Finally, during pregnancy, the alveolar compartment undergoes functional differentiation in response to prolactin and placental lactogens. [370].

Expression of Hh pathway components has been observed during all three stages of mammary gland development. Both Shh and Ihh mRNA are expressed in the developing embryonic mammary epithelium. However, Shh or Ihh null mice exhibit normal mammary gland development [371, 372]. Dhh expression is enriched in the terminal end bud (TEB) epithelium; however, the requirement for Dhh in mammary gland has not been directly analyzed [373]. GLI-1 expression has not been detected during mammary gland development [374]. GLI-2 is expressed only in the stroma during non-parous stages and in both the stroma and the epithelium during pregnancy and lactation. Furthermore, transplantation studies demonstrate that loss of GLI-2 in the stroma leads to irregularities in ductal shapes [375]. GLI-3 is expressed in both the mesenchymal and luminal compartments and loss of GLI-3 leads to inappropriate activation of Hh signaling and failed mammary bud formation [374]. Finally, defects in ciliary assembly result in decreased Hh signaling, decreased branching, and reduced

lobular-alveolar development during pregnancy and lactation [376]. These results suggest that restraining Shh signaling is required for normal mammary gland development.

Mouse models provide further evidence that increased Shh signaling can disrupt mammary gland development. Overexpression of Shh or loss of Smo impairs, and can even block, mammary bud development [377]. Heterozygous loss of Ptch1 or constitutive activation of Smo leads to abnormalities in TEB, which resemble human ductal hyperplasia [378, 379]. Mice expressing the $Ptch1^{mes}$ allele, a spontaneous deletion mutant that associates and activates Src upon Shh binding, exhibit defective ductal elongation and hyperplasia [181, 380]. Mammary epithelial overexpression of GLI-1 leads to reduced alveolar network complexity and impaired lactation [381].

Breast cancer

Breast cancer is the most common cancer affecting women [382]. Breast tumors can be categorized into 18 distinct subtypes based on pathology. The two most common types are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), which represent about 80% and 5% of reported cases respectively [383]. Gene expression profiling has identified distinct biological subtypes of breast cancer: luminal A or B, human epidermal growth factor receptor 2 (HER2) amplified, basal like, and claudin low [384, 385]. The luminal A and B subtypes are both estrogen receptor (ER) positive and comprise up to 70% of all breast cancers. Luminal B tumors are also HER2 positive and have a poorer prognosis [385-387]. The basal like and claudin low subtypes are both triple negative, lacking expression of ER, HER2 and the progesterone receptor (PR).

Estrogen and progesterone are ovarian steroid hormones that play critical roles in the development of the mammary gland. They are also closely linked to breast cancer development and progression because they regulate multiple cell processes such as proliferation and survival [388]. These hormones activate the ligand dependent transcription factor functions of ER and PR, which belong to the nuclear hormone receptor superfamily. ER and PR contain related functional domains: a ligand binding domain, a central DNA binding domain, and transcriptional activation domains AF-1 and AF-2 [389]. AF-1 acts as a ligand independent transcription factor. In contrast, AF-2 is located within the ligand binding domain and regulates ligand dependent transcriptional output. There are two ERs (α and β), which are encoded by two genes on chromosome 6 and 14 respectively. ERα and ERβ share 60% homology and have distinct functions in response to ligand activation. Additionally, each ER has multiple variants [388]. ERα and PR are expressed by 30-50% of luminal cells. In fact, most of these cells express both receptors because PR is an ER target gene [390]. There are two isoforms of PR (A and B), which are transcribed from different promoters of the same gene [391].

In the absence of ligands, both ER and PR are found in complexes with heat shock proteins [392]. Upon ligand binding, the receptors dissociate from these complexes, dimerize, and mediate both genomic and non-genomic signaling. As transcription factors, hormone receptors interact with various co-activator or co-repressor complexes to either activate or repress gene transcription. Alternatively, hormone receptors can interact with each other and with other transcription factors. Furthermore, hormone bound ER and PR can also mediate non-genomic signaling. For example, estrogen bound ER localized to the membrane or cytoplasm can mediate non-genomic

signaling by interacting with Src, PI3K, and G proteins [393]. Similarly, PR can activate Src, MAPK, and Akt signaling. Finally, other signaling pathways – EGFR, HER2, and IGFR – can activate hormone independent ER and PR signaling by phosphorylating various residues on these receptors [388, 394].

HER2 is a member of the epidermal growth receptor family, which also includes EGFR/HER1, ERBB3, and ERBB4 [395]. All family members contain an extracellular ligand binding domain, a single membrane spanning segment, and a cytoplasmic tyrosine kinase domain. Activation of receptors is regulated by the availability of ligands and results in modulation of cell survival, differentiation, and proliferation. EGFR, ERBB3, and ERBB4 are activated upon binding of various ligands. In contrast, no ligand has been identified for HER2. Instead, it is found in a constitutively active conformation. Ligand binding leads to receptor homo- or hetero-dimerization followed by phosphorylation of the kinase domains of EGFR, HER2, and ERBB4. ERBB3 can only activate downstream signaling upon heterodimerization with another ERBB receptor. Activation of these receptors stimulates intracellular signaling through the PI3K/Akt and Ras/Raf/MEK/MAPK pathways. Overexpression of HER2 leads to constitutive activation of these pathways [395, 396].

Treatment of luminal A tumors with tamoxifen, a selective ER modulator, has significantly reduced the mortality rate. However, not all patients respond to tamoxifen and one third of initial responders have recurrent disease within 15 years [397]. Hormone resistance can occur through ER-dependent as well as ER-independent mechanisms, including activation of pro-proliferative signaling pathways such as HER2 and EGFR, PI3K/Akt, and MAPK [398, 399]. Use of trastuzumab, an antibody targeting

HER2, has extended the overall survival of patients with HER2 amplified tumors [400]. However, about 40-60% of these tumors show *de novo* resistance even when treatment is combined with systematic chemotherapy [401]. Furthermore, about 70% of initial responders show progressive disease within a year. Acquired resistance can occur through overexpression of EGFR family receptors or IGF-R1, PTEN loss, or activation of PI3KCA [402-404]. Therefore, there is a need to identify new therapeutic targets.

Key developmental pathways, including Hh, Wnt, and Notch, have also been implicated in the progression of breast tumors. The role of Hh signaling in breast cancer is discussed in detail in the following section. The Wnt family of signaling molecules were discovered as oncogenes in naturally occurring mouse models of breast cancer [405]. Activation of Wnt1, Wnt3, or Wnt10b under the mouse mammary tumor virus (MMTV) promoter leads to the formation of tumors [406-408]. Mutations in the Wnt pathway are uncommon in human breast cancers; however, overexpression of positive regulators or inactivation of negative regulators of Wnt signaling have been identified. For example, Dvl, a positive regulator, is amplified in about 50% of breast cancers [409]. Expression of APC, a negative regulator, is downregulated in about 36-50% of breast cancers [410]. Moreover, mice with APC deletion in mammary progenitor cells develop mammary tumors [411]. Increased expression of β-catenin is found in about 60% of human breast cancer tissue samples and is associated with poor prognosis [412, 413].

A role for Notch in breast cancer was initially identified when Notch was found as a frequent site of MMTV integration [414]. This integration event led to constitutive activation of Notch signaling through the expression of the intracellular domain of Notch4 [415, 416]. Furthermore, transgenic mice overexpressing the intracellular

domains of either Notch1 or Notch3 also develop mammary tumors [417]. Notch4 signaling is highly upregulated in breast cancer stem cells [418]. In humans, overexpression of Notch receptors has been reported in both ductal carcinoma *in situ* (DCIS) and IDC, suggesting increased Notch signaling is an early event in tumor formation [419, 420].

The Hh pathway in breast cancer

Recently, aberrant activation of the Shh pathway has been implicated in breast cancer progression. Initial studies identified rare mutations in Shh and Ptch in breast cancer tissues [326, 421]. Both studies, however, examined only a handful of samples, and analysis of a larger number of tumors failed to identify such mutations [422]. Comparative genomic hybridization analysis revealed low level GLI1 amplifications in 13 out of 31 tumors [423].

Ptch expression is reduced in DCIS, possibly due to increased promoter methylation. In addition, ectopic expression of Smo has been detected in both DCIS and invasive breast cancer. Shh overexpression occurs in breast tumor initiating cells and in IDC, where it is associated with increased metastasis and death [424]. A progressive increase in Shh expression correlates with disease progression from low grade DCIS to IDC [424, 425]. The increase in Shh expression seems to occur through both epigenetic and transcriptional mechanisms. The Shh promoter was found to be methylated in 93.3% of normal breast tissues and only 29.5% of breast cancer tissues [426]. Moreover, the use of alternative transcriptional start sites can induce Shh transcription even in the presence

of promoter methylation [427]. Upregulation of NF-κB, p63, p73, and Runx2 have also been demonstrated to increase Shh or Ihh transcription in breast cancer [426, 428, 429].

Increased expression of Shh, Ptch-1, and Gli-1 was detected in breast cancer cell lines. Although treatment with 10μM cyclopamine reduced cell proliferation [430], use of such a high dose (less than 3μM cyclopamine is sufficient to inhibit Shh signaling [431, 432]) raises concerns of off-target effects. Another study found that GLI-1 expression is higher in ER negative breast cancer cells and that overexpression of GLI-1 in ER positive cells reduces ER transactivation, increases proliferation, and reduces response to anti-estrogen treatment [433]. Breast tumor growth and metastasis in mice is stimulated by Shh overexpression and is decreased by inhibiting Shh signaling. Moreover, GLI-1 expression under the MMTV promoter, leads to the development of hyperplastic lesions and tumors [434].

A number of studies indicate the presence of paracrine Shh signaling in breast cancer, where stromal cells respond to Shh produced by cancer cells. Analysis of ten breast cancer tissues showed increased Shh in the breast cancer epithelium and increased GLI-1 expression in the stroma [435]. Additionally, overexpression of Shh in mouse mammary tumor cells leads to activation of Shh signaling in the stroma [424, 430, 436]. Similarly, while knockdown of endogenous Shh in MDA-MB-231 cells does not alter proliferation *in vitro*, it decreases tumor growth in a xenograft model, suggesting a role for Shh in regulating tumor-stroma interactions [437]. Overexpression of Shh in MDA-MB-231 cells enhances proliferation, anchorage-independent growth, and invasion. In addition, Shh expressing MDA-MB-231 cells show increased expression of CYR61 (Cysteine-rich angiogenic inducer 61) and form larger, more vascularized tumors,

suggesting a role in the tumor-microenvironment interaction [438]. Furthermore, patients with advanced breast cancer show increased expression of both Shh and CYR61 [438].

Various studies have indicated a role for Hh signaling between cancer cells and osteoclasts. First, Shh and Ihh secreted by breast cancer cells can increase osteopontin (OPN) transcription in pre-osteoclasts, thereby inducing osteoclast differentiation and increasing bone resorption [439]. Conversely, treatment of breast cancer cells with OPN results in increased GLI-1 transcription, which promotes expression of mesenchymal markers and increases multidrug resistance. Treatment with GANT61, a GLI1/2 antagonist, reverses these phenotypes [440]. GLI-2 expression in cancer cells increases PTHrP secretion leading to increased bone destruction and metastases in tumor bearing mice [441]. Overexpression of a GLI-2 repressor construct in cancer cells results in smaller osteolytic lesions. In contrast, cyclopamine treatment does not reduce GLI-2 expression, alter tumor growth or bone metastasis, suggesting non-canonical regulation of GLI-2 expression. Indeed, TGFβ was found to induce GLI-2 expression [442].

Hh signaling in mammary and tumor stem cells

Two groups initially isolated mammary stem cells based on the expression of cell surface markers – $CD45^{-}Ter119^{-}CD39^{-}Sca\text{-}1^{low}CD24^{med}CD49f^{high}$ [443, 444]. This rare population of cells is capable of generating an entire mammary gland when transplanted into a cleared fat pad. Further examination revealed that both luminal and myoepithelial lineages contain long lived unipotent stem cells with extensive renewing capacities [445]. Human derived mammosphere cultures, which enrich for mammary stem progenitor cells, exhibit significantly upregulated expression of Ihh, Ptch-1, Smo, Gli-1 and Gli-2

[364]. Additionally, treatment with recombinant Shh increases mammosphere number and size, whereas treatment with cyclopamine has the opposite effect. Furthermore, the mammary progenitor cell compartment is expanded in $Ptch^{+/-}$ mice [446].

Isolated breast CSCs express higher levels of Ptch-1, Gli-1 and Gli-2 [364]. Additionally, Gli-1 overexpressing tumors exhibit an expansion of tumor progenitor cells [434]. Tumor stem cells are often resistant to therapy and can repopulate the tumor after treatment. A recent study found that treatment of breast cancer cells with docetaxel leads to increased Hh signaling. Furthermore, Hh signaling is required for the expansion of stem like population upon docetaxel treatment [447]. Taken together, these data support a role for Shh signaling in maintaining the breast CSC population.

Targeting the Hedgehog pathway in cancer

Constitutive activation of Hh signaling is involved in the progression of numerous tumors. Therefore, molecules that antagonize Hh signaling are in development for the treatment of these cancers. Most of these compounds are Smo inhibitors in various stages of clinical development [448]. For example, vismodegib was recently approved for the treatment of BCCs after showing 43% and 30% response rates in locally advanced and metastatic BCC, respectively, in clinical trials [449]. Other Smo inhibitors – sonidegib, BMS-833923, IPI-926 – also showed promising results in clinical trials with BCC patients, suggesting these tumors are especially sensitive to Smo inhibition [448]. In addition, molecules which abrogate cilial translocation of Smo have emerged as another method of reducing Shh signaling. Most of these molecules interact directly with Smo

while others interfere with ciliogenesis or modulate Smo translocation through unknown mechanisms. For these molecules, it is critical to determine the mechanism of action in order to identify and mitigate possible side effects [448]. While Smo inhibitors are generally well tolerated by patients, resistance to these inhibitors is common. Resistance has been shown to occur through various mechanisms such as mutations in Smo, amplifications of Gli2, or upregulated Gli activation through other signaling pathways [448].

Drugs already in use for various other indications have also emerged as modulators of Hh signaling. For instance, the effect of glucocorticoids on Shh signaling has recently been elucidated. Interestingly, one class of glucocorticoids promotes hypersensitivity to Hh stimulation while another class inhibits Hh signaling. The latter class of glucocorticoids inhibits cilial accumulation of Smo and therefore inhibits the activity of not just wild type but also mutant Smo proteins, which do not respond to other Smo antagonists [450]. Another established drug, intraconazole, was recently found to inhibit Hh signaling. This antifungal drug was shown to prevent Smo translocation and inhibit Hh driven MB and BCC growth in mice [451, 452]. In addition, it was shown to inhibit metastatic prostate cancer growth in a clinical trial [453]. Finally, arsenic trioxide (ATO), which is currently used to treat acute promyelocytic leukemia, was also shown to reduce Hh driven cancer growth. The precise mechanism of action remains uncertain. In one study, ATO was found to inhibit the Hh induced accumulation of GLI2 in cilia, and ATO treatment reduced the growth of $Ptch^{+/-}p53^{-/-}$ MB allografts in a dose dependent manner [454]. A second study found that ATO reduced the growth of Ewing sarcoma xenografts by directly binding GLI1 and reducing GLI1 transcriptional output [455].

Inhibitors which target GLI transcriptional activation have also been identified for potential use as therapeutics in Hh driven cancers. Two structurally distinct compounds, GANT58 and GANT61, reduce GLI mediated transcription and therefore decrease proliferation of various Hh dependent tumor cells [456]. In addition, both compounds decrease the growth of prostate cancer xenografts in nude mice. GANT61 was found to directly disrupt GLI-DNA interactions. Another screen identified several compounds, Hh pathway inhibitors (HPI 1-4), which interfere with Hh signaling through different mechanisms. HPI1 inhibits GLI1/2 activity independently of the cilium, HPI2 and 3 inhibit GLI2 processing and activation, and HPI4 disrupts primary cilia formation [457]. These inhibitors are promising for tumors in which Gli transcription is upregulated by Hh or other pathways.

Finally, because Shh can have both Smo dependent and independent effects, inhibiting Shh signaling at the level of the ligand provides an effective method of disrupting both canonical and non-canonical signaling. Robotnikinin is a small molecule that directly binds to purified ShhN and interferes with the Shh-Ptch interaction. Co-treatment of cells with robotnikinin and Shh reduces Shh induced GLI luciferase reporter activity and endogenous GLI2 expression [458]. Alternatively, a novel method of inhibiting Hh signaling relies on interfering with the processing of Hh ligands. Our laboratory conducted a screen for small molecule inhibitors of Hhat, and identified a number of structurally related compounds, including RU-SKI 43. RU-SKI 43 inhibits Hhat mediated palmitoylation of Shh and reduces autocrine and paracrine Shh signaling. Importantly, RU-SKI 43 does not interfere with fatty acylation of other proteins including

HRas, Fyn, or Wnt [459]. Furthermore, RU-SKI 43 reduces the proliferation of pancreatic cancer cells both *in vitro* and *in vivo* [24].

Topological organization of Hhat

While recent studies have demonstrated an important role for Hhat in the progression of pancreatic and lung cancers [24-27], structural information for Hhat is lacking. The crystal structure of Hhat has not yet been determined, and there are considerable difficulties associated with overexpression and crystallization of membrane proteins [460]. A fundamental aspect of membrane protein structure is topology, the number of transmembrane domains (TMDs) and their orientation with respect to the membrane. Knowledge of the transmembrane topology of Hhat could aid in the further design of selective Hhat inhibitors. The determination of Hhat topology is discussed in Chapter Three.

TMD helices are connected by hydrophilic loops, which are usually the most flexible parts of membrane proteins [461, 462]. Initial studies suggested that TMDs are mainly hydrophobic α-helices oriented more or less perpendicular to the membrane. However, with increasing availability of structural data, it has become clear that short, long, tilted, kinked, marginally hydrophobic, re-entrant and interfacial TMD helices exist [463-465].

Most membrane proteins are co-translationally inserted into the membrane by a translocon, a process which determines the topological organization of the protein [465]. The best characterized translocons are the Sec61 translocon, which resides in the rough

endoplasmic reticulum, and its bacterial homolog SecYEG. The Sec61 translocon has two functions. First, it forms an aqueous channel which allows entry of secretory proteins into the secretory pathway. Second, it opens laterally into the lipid bilayer and allows TMDs to enter sideways into the bilayer [466-471].

Structural analysis revealed that the Sec61 translocon has an inverted U shape structure with an opening into the bilayer [472]. This opening is termed the 'lateral gate' and allows TMDs to sample their environment during the insertion process. The translocon complex consists of two heterotrimeric copies of Sec61, arranged in a back-to-back configuration, and two copies of the tetrameric translocon-associated protein (TRAP) [473, 474]. It seems that only one of the SecY/Sec61 heterotrimers is involved in the insertion process at any given time [475]. In addition, a number of proteins are closely associated with the translocon complex: the translocating chain-associated membrane protein (TRAM); the signal peptidase complex (SPC), which identifies and cleaves the signal sequences; and the oligosaccharyl transferase (OST), which glycosylates the -Asn-X-Ser/Thr- consensus site on secreted and membrane proteins [476-479].

Disulfide cross-linking experiments demonstrated how each TMD in a nascent polypeptide exits the translocon and is integrated within the lipid bilayer [480]. Each TMD enters the membrane in an N-to-C succession. Importantly, certain TMDs can re-enter the translocon channel when a downstream helix enters the translocon. This may influence the insertion process by allowing the two helices to interact. In fact, "marginally hydrophobic" TMDs (mTMDs) cannot be inserted into the membrane when

expressed on their own. Rather, they require the presence of adjacent helices for efficient integration into the lipid bilayer [480].

Certain polypeptide sequences are efficiently integrated into the lipid bilayer with a preferred orientation. In fact, for single spanning membrane proteins, a "threshold hydrophobicity" determines whether or not a polypeptide segment inserts into the membrane [481]. In contrast, such a threshold does not seem to exist for multipass proteins. For example, a number of mTMDs have been identified in multipass proteins. For these sequences, the adjacent loops or upstream/downstream TMDs are required for proper membrane insertion [460, 482]. Alternatively, hydrophobic stretches can also be excluded from membranes. For example, the citrate transporter CitS has a hydrophobic segment that is translocated to the periplasm, a process which requires the presence of the preceding TMD helix [483]. The fact that TMDs can influence the insertion or exclusion of other hydrophobic segments suggests that two or possibly three TMs can remain within the translocation channel. In fact, the multidrug transporter P glycoprotein is integrated into the membrane only upon completion of synthesis [484]. Alternatively, TMDs may form folding intermediates after lateral movement into the bilayer and be inserted into the membrane with the help of chaperone-like proteins [485, 486].

Analysis of 46 α-helical membrane protein structures revealed the distribution and preferred location of each amino acid in reference to the membrane lipid bilayer [460]. In general, hydrophilic residues prefer aqueous domains and hydrophobic residues prefer the transmembrane region. Specifically, charged residues – Arg, Asp, Gln, and Lys – avoid the transmembrane region and are concentrated at the interface of the membrane and aqueous environment. Moreover, positively charged residues are preferentially

enriched in cytoplasmic loops. Hydrophobic residues – Ala, Ile, Val, and Leu – prefer the transmembrane region. Additionally, Ala and Gly prefer loops and core regions of α-helices as well as in re-entrant loops [487, 488]. Polar residues show differential preferences: Asp and Glu avoid membrane regions; Ser and Thr are evenly distributed throughout the protein; and Pro and Gly are enriched at interfacial loop regions and membrane regions. Pro and Gly residues are thought to increase the stability by interlocking helices or provide molecular hinges for conformational transitions [489, 490]. Aromatic residues – Trp and Tyr– are enriched in interfacial helices while Phe, similar to hydrophobic residues, is present throughout the membrane region [460, 491].

Information from experimentally determined topologies and structures has been used to derive algorithms that predict membrane protein topology from amino acid sequences. Initial prediction methods relied on two observations (i) TMD α-helices tend to be highly hydrophobic, and (ii) the hydrophilic loops connecting TMDs tend to be enriched for positively charged residues on the cytoplasmic side (the positive inside rule) [492]. In eukaryotes, however, the tendency for the positive charges to be absent from periplasmic/lumenal domains is weaker [493]. Incorporation of machine-learning techniques such as neural networks and hidden Markov models in algorithms have significantly improved prediction programs [494-497].

Certain prediction programs have also included information from *in vitro* studies which quantified the contribution of individual amino acids to the free energy of membrane insertion. For these studies, a panel of model hydrophobic segments (H-segments), flanked by two N-linked glycosylation sites, was generated. Because glycosylation occurs in the lumen, the insertion efficiency and apparent free energy

(ΔG_{app}) of membrane insertion can be calculated for individual H-segments. This method was used to analyze the positional effect of each amino acid on membrane insertion efficiency, and the information was used to develop topology predictor programs [481, 498].

Experimental determination of topology

Cytoplasmic and lumenal portions of membrane proteins are separated by a lipid bilayer and are therefore differentially accessible to various reagents. A variety of molecular and biochemical techniques take advantage of this fact to experimentally determine the topology of membrane proteins. Using these assays, one can infer the topology of a given hydrophilic loop based on the localization of an epitope tag, glycosylation sequence, or a labeled Cys residue.

Epitope tags are commonly used to determine topological organization. Initial studies relied on C-terminal truncations with an epitope tag [499, 500]. The orientation of the C-terminus can be inferred from the orientation of the epitope tag. However, this method is limited because truncations may alter the stability or overall topology of the protein. If, for example, a TMD requires a downstream TMD for efficient insertion, then a truncated protein would give a false readout. An alternative approach is to insert epitope tags within various hydrophilic loops of the protein. In this case, the relatively small tag may be less likely to alter the overall topology.

Two assays which are commonly used with epitope tag insertion proteins rely on either protease protection or antibody accessibility. For protease protection assays, cells

are transfected with each epitope insertion construct and microsomal membranes are prepared. These membrane preparations are oriented primarily right-side-out (i.e. cytoplasmic surface on the outside). Membranes are then treated with or without trypsin in the presence or absence of detergent. In the absence of detergent, cytoplasmic epitope tags are exposed and therefore digested by trypsin whereas lumenal tags are protected by the membrane. The addition of detergent permeabilizes the membranes thereby allowing trypsin to access lumenal epitope tags. Protein disulfide isomerase (PDI), a lumenal endoplasmic reticulum marker, can be used to confirm the right-side-out orientation of the membranes and serve as an internal control of a lumenal epitope. Protease protection assays, used in conjunction with other assays, have been used to determine the topology of the γ-secretase component PEN-2, stearoyl-CoA desaturase-1, ACAT2, DGAT1 and DGAT2 [278, 501-504].

Epitope insertion constructs can also be used in immunofluorescence based assays that rely on selective permeabilization of cellular membranes [278, 501-504]. In this assay, cells transfected with individual constructs are grown on coverslips. Cells are treated with digitonin, to selectively permeabilize the plasma membrane, or with TritonX-100, to permeabilize all cellular membranes. Cells are then incubated with an antibody directed against the epitope tag or control antibody and visualized with confocal microscopy. In digitonin treated cells, only cytoplasmic tags are detected by the antibody. In Triton X-100 treated cells, both cytoplasmic and lumenal epitope tags can be detected.

Glycosylation scanning mutagenesis is another method used to determine topological organization of proteins [501, 505]. In this assay, the localization of the loop in the lumenal or cytoplasmic side of the membrane is inferred from the presence or

absence of glycosylation on an engineered protein. In eukaryotic cells, glycosylation is mediated by OST in the lumen of the endoplasmic reticulum. OST adds oligosaccharides to the amino group of an Asn residue in the Asn-X-Thr/Ser sequence [506]. The catalytic site of OST faces the lumen of the endoplasmic reticulum and the glycosylated Asn residue must be located a minimum of 12 residues upstream or 14 residues downstream of a TMD segment [506, 507]. Not surprisingly, glycosylation occurs only in loops longer than 25 residues [508, 509]. To determine topology of a given protein, the glycosylation consensus sequence can be introduced into loops longer than 25 residues. A lumenal loop is expected to be glycosylated and cause an upward shift in gel mobility (about 2.5kDa), which should be blocked upon digestion with a glycosidase [483, 510, 511]. Glycosylation of proteins can be assessed directly in cell lysates or coupled with *in vitro* transcription/translation assays. It is important to note that lack of glycosylation does not always indicate a cytoplasmic orientation. In fact, lumenal loops are not always glycosylated [511].

Finally, cysteine scanning mutagenesis, which relies on chemical modification of Cys residues with sulfhydryl reagents, can be used to determine the orientation of Cys residues and therefore the corresponding loop within a given protein [502, 512]. First, single Cys mutants are generated by mutating all but one Cys residues within a protein to Ser, which often has minimal impact on protein activity. Cys residues that are labeled with a sulfhydryl reagent after selective plasma permeabilization indicate a cytoplasmic orientation. On the other hand, Cys residues that are labeled only after full permeabilization indicate a lumenal orientation. Sulfhydryl reagents containing a biotin group, a fluorescent group, or a radiolabel allow for detection of the labeled protein [513-

515]. The location of the Cys residue needs to be carefully chosen as Cys residues close to putative TMDs are less reactive with biotin containing reagents [514]. Cysteine scanning mutagenesis is a useful technique because analysis is performed in whole cells and chemical modification of Cys residues occurs after insertion into the membrane.

Membrane topologies of MBOAT enzymes

The transmembrane topologies of several mammalian MBOAT enzymes have been experimentally determined. The topology of human ACAT1 was initially investigated using selective permeabilization and inserted epitope tag constructs [516]. The N-terminus is oriented toward the cytosol whereas the C-terminus is oriented within the lumen of the endoplasmic reticulum. The authors identified seven TMDs, however, they noted the presence of a highly hydrophobic segment in the C-terminal portion of the protein, suggesting the presence of additional TMDs. The topology of ACAT1 was later re-examined with a cysteine scanning mutagenesis assay [517]. This approach identified two additional TMDs and placed the active site His residue within the endoplasmic reticulum membrane.

Two models have been proposed for the topological organization of ACAT2. In one model, ACAT2 contains five TMDs with the N-terminus in the cytoplasm and the C-terminus in the lumen of the ER [518]. In a second model, ACAT2 was proposed to contain only two TMDs, with the active site His residue embedded within a re-entrant loop and both termini located in the cytoplasm [278]. However, in this model, there are at least five additional hydrophobic segments which may form potential interfacial helices

or reentrant loops. The discrepancy between the two models may be due to differences in their approaches; Joyce and colleagues [518] relied on the use of truncated constructs whereas Lin and colleagues [278] used epitope insertion constructs.

The topological architecture of murine DGAT1 and DGAT2 were identified using protease protection and selective permeabilization assays. While DGAT1 is predicted to have eight TMDs, biochemical analyses verified only three TMDs. The N-terminus of DGAT1 was found to be oriented in the cytosol whereas the C-terminus was oriented in the lumen of the endoplasmic reticulum [503]. DGAT2 contains only two closely spaced TMDs within the N-terminal portion of the protein, with both N- and C-termini localized within the cytoplasm [504].

Within the mammalian protein acyltransferase subfamily of MBOAT enzymes, only the topology of GOAT has been extensively examined. GOAT contains eleven TMDs and one re-entrant loop [282]. The active site His residue is located within a TMD near the lumen of the endoplasmic reticulum, while the highly conserved Asn residue is located in a large loop within the cytosol. The topology of GOAT resembles that of yeast Gup1p [519] as both enzymes have 12 membrane embedded regions connected by short hydrophilic loops. The topology of Hhat, and the similarities with GOAT, is discussed in Chapter Three.

Chapter Two

Hedgehog Acyltransferase as a target in estrogen receptor positive, HER2 amplified, and tamoxifen resistant breast cancer cells

Breast cancer is the most common cancer affecting women [382]. Gene expression profiling has identified distinct biological subtypes of breast cancer: luminal A or B, human epidermal growth factor receptor 2 (HER2) amplified, basal like, and claudin low [385]. The luminal A and B subtypes are both estrogen receptor (ER) positive and comprise up to 70% of all breast cancers. Luminal B tumors are also HER2 positive and have a poorer prognosis [385-387]. The basal like and claudin low subtypes are both triple negative, lacking expression of ER, HER2 and the progesterone receptor. Treatment of luminal A tumors with tamoxifen, a selective ER modulator, has significantly reduced the mortality rate. However, not all patients respond to tamoxifen and one third of initial responders have recurrent disease within 15 years [397]. Hormone resistance can occur through ER-dependent as well as ER-independent

mechanisms, including activation of pro-proliferative signaling pathways such as HER2 and EGFR [398], PI3K/Akt, and MAPK [399]. Use of trastuzumab, an antibody targeting HER2, has extended the overall survival of patients with HER2 amplified tumors [400]. However, about 40-60% of these tumors show *de novo* resistance even when treatment is combined with systematic chemotherapy [401]. Furthermore, about 70% of initial responders show progressive disease within a year. Acquired resistance can occur through overexpression of EGFR family receptors [402] or IGF-R1 [403], PTEN loss, or activation of PI3KCA [404, 520]. Therefore, there is a need to identify new therapeutic targets.

Recently, aberrant activation of the Sonic Hedgehog (Shh) pathway has been implicated in breast cancer progression [182, 422, 424-426, 430, 434-436, 439, 440, 442, 521]. The hedgehog family of secreted signaling molecules includes Shh, Indian and Desert Hedgehog. Interaction of Shh with the transmembrane receptor Patched-1 (Ptch-1) relieves inhibition of the transducer Smoothened (Smo). This leads to the stabilization and nuclear translocation of the Gli family of transcription factors [11]. The resulting activation of target gene transcription regulates various cellular processes such as cell fate determination, proliferation, and survival [11]. A role for abnormal Shh signaling activity in breast cancer development was first reported using transgenic mouse models, where Ptch-1 haploinsufficiency or ectopic expression of Smo lead to distinct forms of mammary ductal dysplasia [378, 379]. Furthermore, expression of Gli-1 under the mouse mammary tumor virus promoter leads to the development of hyperplastic lesions and tumors [434]. Mutations in Shh, Ptch, and Smo are rarely identified in human breast cancer [422]. Ptch expression is reduced in ductal carcinoma *in situ* (DCIS) [378, 522],

possibly due to increased promoter methylation [522]. In addition, ectopic expression of Smo has been identified in both DCIS and invasive breast cancer [378]. Breast tumor growth and metastasis in mice is stimulated by Shh overexpression and is decreased by inhibiting Shh signaling [424]. In humans, Shh overexpression occurs in breast tumor initiating cells and in invasive ductal carcinoma (IDC), where it is associated with increased metastasis and death [424]. A progressive increase in Shh expression correlates with disease progression from low grade DCIS to IDC [424, 425]. In addition, three studies have noted strong Gli-1 expression in stromal cells [424, 430, 435]. Shh and Ihh secreted by breast cancer cells can signal in a paracrine manner to induce osteoclast differentiation and increase bone resorption [439]. Furthermore, other pathways, including osteopontin and TGFβ, can also activate Gli-mediated transcription in breast cancer cells [440, 442].

To date, analyses of the hedgehog pathway in breast cancer have focused mainly on downstream signaling events. Little is known about components of the pathway upstream of ligand production. Shh is synthesized as a precursor protein that undergoes autoprocessing to produce a ~25kDa C-terminal fragment and a ~19kDa N-terminal fragment (ShhN) that retains all signaling activity [14, 15]. ShhN is modified with two lipids. Cholesterol is covalently attached to the C-terminus during the autoprocessing reaction [16]. Cholesterol attachment contributes to long-range signaling activity, but is not essential for signaling [17]. The N-terminus of ShhN is modified by covalent attachment of the 16-carbon fatty acid palmitate to the N-terminal cysteine [18, 19]. Shh palmitoylation is catalyzed by Hedgehog acyltransferase (Hhat), a multipass transmembrane enzyme that belongs to the membrane bound O-acyltransferase

(MBOAT) family [19]. Multiple studies have established that palmitoylation of Shh by Hhat is critical for Shh signaling activity [17, 20-23]. Furthermore, Hhat activity is required for the proliferation of pancreatic cancer cells *in vivo* and for the maintenance of a stem-like phenotype in lung squamous cell carcinoma [24-27].

The role of Hhat in breast cancer has not yet been examined. In this study, we demonstrate that Hhat is required for the proliferation of ER positive, HER2 positive, and tamoxifen resistant breast cancer cells. Increased Hhat expression resulted in increased cell proliferation, while Hhat depletion reduced proliferation of ER positive cells. Hhat inhibition with RU-SKI 43, a selective small molecule inhibitor of Hhat recently identified by our group [459], also reduced the growth of ER positive cells. Furthermore, Hhat depletion or inhibition led to a significant decrease in HER2 positive and tamoxifen resistant cell proliferation. None of the cell lines we tested responded to inhibition of Smo, and only a subset responded to Shh depletion, indicating that non-canonical Shh signaling pathways were operative. Taken together, these data suggest that Hhat may serve as an important therapeutic target in ER positive, HER2 amplified, and hormone resistant breast cancers.

Experimental Procedures

Reagents and antibodies – Lipofectamine 2000® and TRIzol® were obtained from Invitrogen (Carlsbad, CA). Polybrene was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-HA antibodies, 17β-estradiol, 4-hydroxytamoxifen, and puromycin were purchased from Sigma (St. Louis, MO). Anti-actin was purchased from

BD Bioscience (San Jose, CA). The ErbB2/HER2, ERα, and pSer118 ERα antibodies were purchased from Cell Signaling (Danvers, MA). LDE-225, LY2940002, and lapatinib ditosylate were purchased from Selleckchem (Houston, TX). Rapamycin was obtained from Fisher Scientific (Waltham, MA). Blasticidin S Hydrochloride was obtained from MP Biomedicals (Santa Ana, CA). 0.4% Trypan Blue Solution was purchased from Cellgro (Manassas, VA). Recombinant human Shh(C24II) was purchased from R&D Systems (Minneapolis, MN).

Plasmids – Plasmids encoding short hairpin RNA (shRNA) sequences for Shh (Clone IDTRCN0000033304), Hhat shRNA 1 (Clone ID TRCN0000035600) and Hhat shRNA 2 (Clone ID TRCN0000035601), cloned into the pLKO.1 vector, were purchased from Open Biosystems (Lafayette, CO). Control pLKO.1 vector, carrying a scrambled shRNA sequence, as well as pHRD8.2 and pCMV VSV-G plasmids, were gifts from Dr. Filippo Giancotti (Memorial Sloan Kettering Cancer Center, New York, NY). The pLenti6/V5-GW/lacZ vector was purchased from Invitrogen (Carlsbad, CA).

Cell culture – Human breast cancer cell lines were gifts from the following colleagues at Memorial Sloan Kettering Cancer Center, New York, NY: T47D, HCC1428, BT474 (Dr. Jacqueline Bromberg), MCF7 (Dr. Michael Overholtzer), BT549 and MDA-MB-231 (Dr. Alan Hall), Hs578t, CAMA-1, MDA-MB-453, and SK-BR-3 (Dr. Filippo Giancotti). Cells were grown following ATCC guidelines. TamR cells were a gift from Dr. Guangdi Wang (Xavier University of Louisiana, New Orleans, LA) and grown in ATCC-

formulated Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS and 1.0 x 10^{-4} M 4-hydroxytamoxifen. All cell lines were authenticated by the ATCC/Promega Cell Line Authentication Service using Short Tandem Repeat profiling analysis performed on July 1, 2014. All cell lines were scored as an exact match for the corresponding ATCC human cell line except for the TamR cell line, which was a 93% match to parental MCF7 cells.

Lentivirus production and knockdown – Endogenous Shh or Hhat were depleted using shRNA delivered to cells via a lentiviral system. Target sequences are: Shh shRNA(CTACGAGTCCAAGGCACATAT), control scrambled shRNA(CCTAAGGTTAAGTCGCCCTCG),Hhat shRNA 1 (GCCACATGGTAGTGTCTCAAA) and Hhat shRNA 2 (CGTGAGCACCATGTTCAGTTT). The shRNA-expressing lentiviruses were produced by co-transfecting confluent 293T cells in 15cm plates with the pLKO.1 shRNA plasmid, the HIV packaging vector pHRD8.2, and pcDNA3.1 VSV-G, using Lipofectamine2000®. Virus was collected 48 and 72 h later as follows. First, media was cleared of debris by centrifugation at 500xg for 5 min. Next, the supernatant was filtered through a 0.45µm filter, and centrifuged at 38720 x g for 2h at 4ºC in SS-34 Rotor on RC6C centrifuge (Sorvall, Asheville, NC). Finally, the pelleted virus was resuspended in ATCC-formulated Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS, and stored at -80ºC. Transduction of cells with lentiviruses was carried out in the presence of 6µg/ml Polybrene. Stable cell lines were produced by transducing target cells

with either control scrambled, Shh, or Hhat shRNA expressing lentiviruses, followed by selection in puromycin.

Hhat overexpression – The pLenti6/V5-GW/lacZ vector was purchased from Invitrogen. The lacZ gene was removed by digestion with SpeI and XhoI, and HhatHA flanked by SpeI and XhoI sites was ligated into the vector. All constructs were confirmed by DNA sequencing. Lentivirus was produced as above and stable cell lines were generated by transducing target cells with either LacZ or HhatHA expressing lentiviruses. Cells were selected in Blasticidin S.

Anchorage dependent cell proliferation – Cells were plated in 6-well plates (0.5-1 x 10^5 cells/well, depending on cell type). For experiments involving drug treatment, drugs were added to the media 24h after plating and media was refreshed every 48h. Cells were grown for up to 6 days, trypsinized and counted with a hemocytometer.

Anchorage independent cell proliferation – Cells were plated in Corning Costar Ultra-Low attachment 24-well plates (0.1-0.2x10^5 cells/well). For experiments involving drug treatment, drugs were added to the media 24h after plating and replenished every 48h. After 14 days, cells were pelleted, washed with PBS, and treated with 0.05% Trypsin-EDTA. The trypsin was quenched with cell culture media, 0.4% Trypan Blue Solution was added and cells were counted with a hemocytometer.

qRT-PCR – Total RNA was isolated using TRIzol extraction. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. qRT-PCR was used to determine expression levels of Hhat, Shh, Ihh, Dhh, Patched-1, Patched-2, hHIP, Smoothened, Gli-1, Gli-2, Gli-3 and HPRT using SsoAdvanced™ SYBR® Green Supermix and the CFX Connect Real Time System (Bio-Rad Laboratories, Hercules, CA). Gene specific primers are listed in Additional file 6: Table S1. Hypoxanthine Phosphoribosyltransferase 1 (HPRT) was used as an endogenous reference, and the relative expression levels of each gene were normalized using the comparative Ct method. Gene expression was normalized to the endogenous reference given by $2^{-\Delta\Delta CT}$.

Immunoblot analysis – Cells were lysed in radioimmune precipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA). Lysates in sample buffer were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes, and probed with the indicated antibodies. To monitor phosphorylation of ERα Ser118, MCF7 or TamR cells were treated with either DMSO or 10μM RU-SKI 43 for 4 h. Media was also supplemented with either ethanol or 200nM 17β-estradiol for the last 30 min of incubation. Cells were lysed in RIPA buffer containing Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates in sample buffer were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes, and probed with the indicated antibodies.

Indirect Immunofluorescence – MCF7 cells were seeded onto coverslips in 6-well plates and cultured for an additional 24 h. Cells were treated with either DMSO or 10μM RU-SKI 43 for 4 h. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Cells were incubated with anti-ERα (Cell Signaling) for 1 h followed with incubation with a secondary antibody (Alexa Flour® 488-conjugated anti-mouse IgG) for 45 min. Slides were mounted with ProLong® Gold Antifade (Invitrogen). Images were collected using a Leica SP5 confocal microscope and analyzed with the Leica Application Suite software. Images were collected using the same conditions on the same day ensuring fair side-by-side comparison.

Results

Hhat depletion results in reduced ER positive breast cancer cell proliferation **–** To investigate the role of Hhat in breast cancer, we used a panel of ER positive (T47D, MCF7, HCC1428, CAMA-1, and BT474) and ER negative (MDA-MB-231, BT549, Hs578t, and MDA-MB-453) cell lines. ER and HER2 expression status was verified in the above cell lines (Fig. 2.1). Hhat mRNA was detected in all cell lines to varying degrees, with mostly higher expression in the ER positive cells (Fig. 2.2A). To assess the functional significance of Hhat expression in breast cancer cells, two different lentiviral based short hairpin RNAs were used to stably deplete Hhat mRNA. Hhat depletion (Fig. 2.3A) led to a 66% reduction in proliferation of ER positive T47D cells, compared to the scrambled shRNA control (Fig. 2.2B). Similar results were observed in all ER positive

cell lines (Fig. 2.2C-F, Fig. 2.3B-E). By contrast, depletion of Hhat in triple negative cells (Fig. 2.3G-I) did not alter cell proliferation (Fig. 2.2H-J). We then monitored anchorage independent growth, a hallmark of neoplastic cells. Hhat depletion in ER positive, but not in triple negative, cells resulted in markedly reduced anchorage independent proliferation (Fig. 2.4A-F). These data indicate that Hhat regulates anchorage dependent and independent proliferation of ER positive cells.

Hhat inhibition leads to decreased ER positive breast cancer cell proliferation – To validate that Hhat activity is required for ER positive breast cancer cell growth, we used RU-SKI 43, a selective small molecule inhibitor of Hhat previously identified by our laboratory [459]. Treatment of T47D cells, which express relatively high levels of Hhat, with increasing concentrations of RU-SKI 43 resulted in a dose dependent decrease in cell proliferation (Fig. 2.5A). Moreover, Hhat inhibition also significantly reduced proliferation of all ER positive cells tested (56-95% depending on cell type) but had no effect on triple negative cells (Fig. 2.5B). Importantly, C2, a compound that is structurally related to RU-SKI 43 but does not inhibit Hhat activity [459], did not affect breast cancer cell proliferation (Fig. 2.5C). The growth defect induced by RU-SKI 43 was rescued, in part, by Hhat overexpression (Fig. 2.5D-F). These data indicate that Hhat inhibition by RU-SKI 43 reduces ER positive cell proliferation.

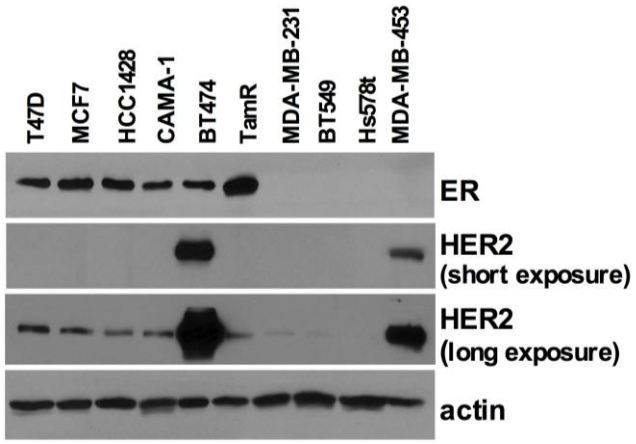

Figure 2.1 ER and HER2 expression in breast cancer cell lines. Cell lysates from indicated breast cancer cells were analyzed directly by Western blotting for ER and HER2 expression. The experiment was performed three times using cells at three different passages.

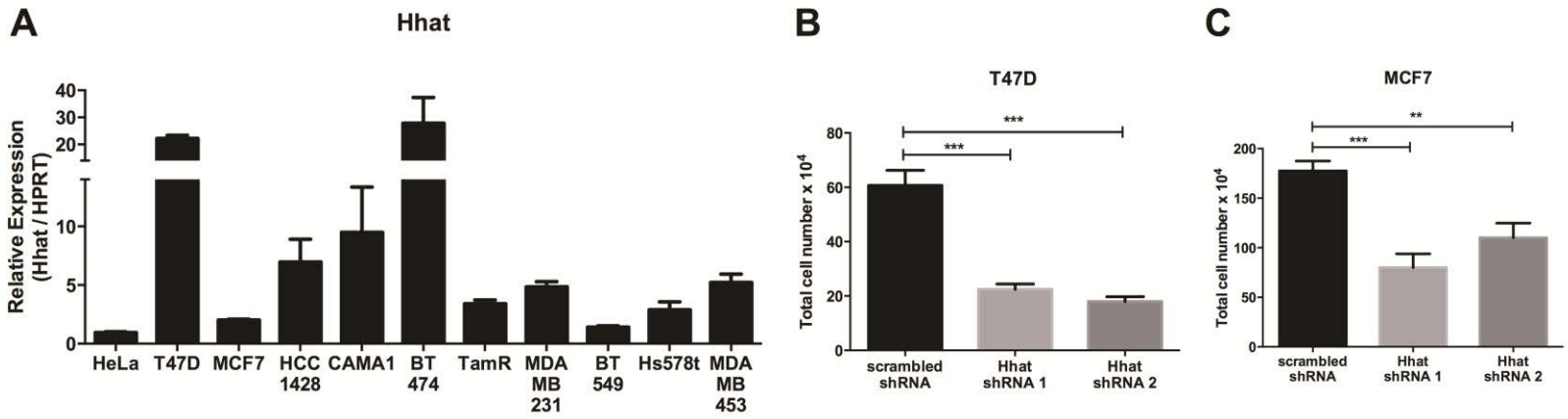

Figure 2.2 Hhat depletion reduces proliferation of ER positive breast cancer cells. A, Hhat mRNA expression in indicated breast cancer cell lines and a control cervical cancer (HeLa) cell line, was measured by qRT-PCR. Hhat expression is shown relative to the expression in HeLa cells, which is set to 1. Bars represent mean ± SD (n=3). Experiments were performed twice in triplicate. B-C, Total cell number at day 6 for (B) T47D and (C) MCF7 cells stably expressing scrambled or Hhat shRNAs were seeded at 5-7x10^4 cells/well, depending on cell type, in 6-well plates and cell numbers were quantified on day 6. Bars represent mean ± SD (n=3). Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

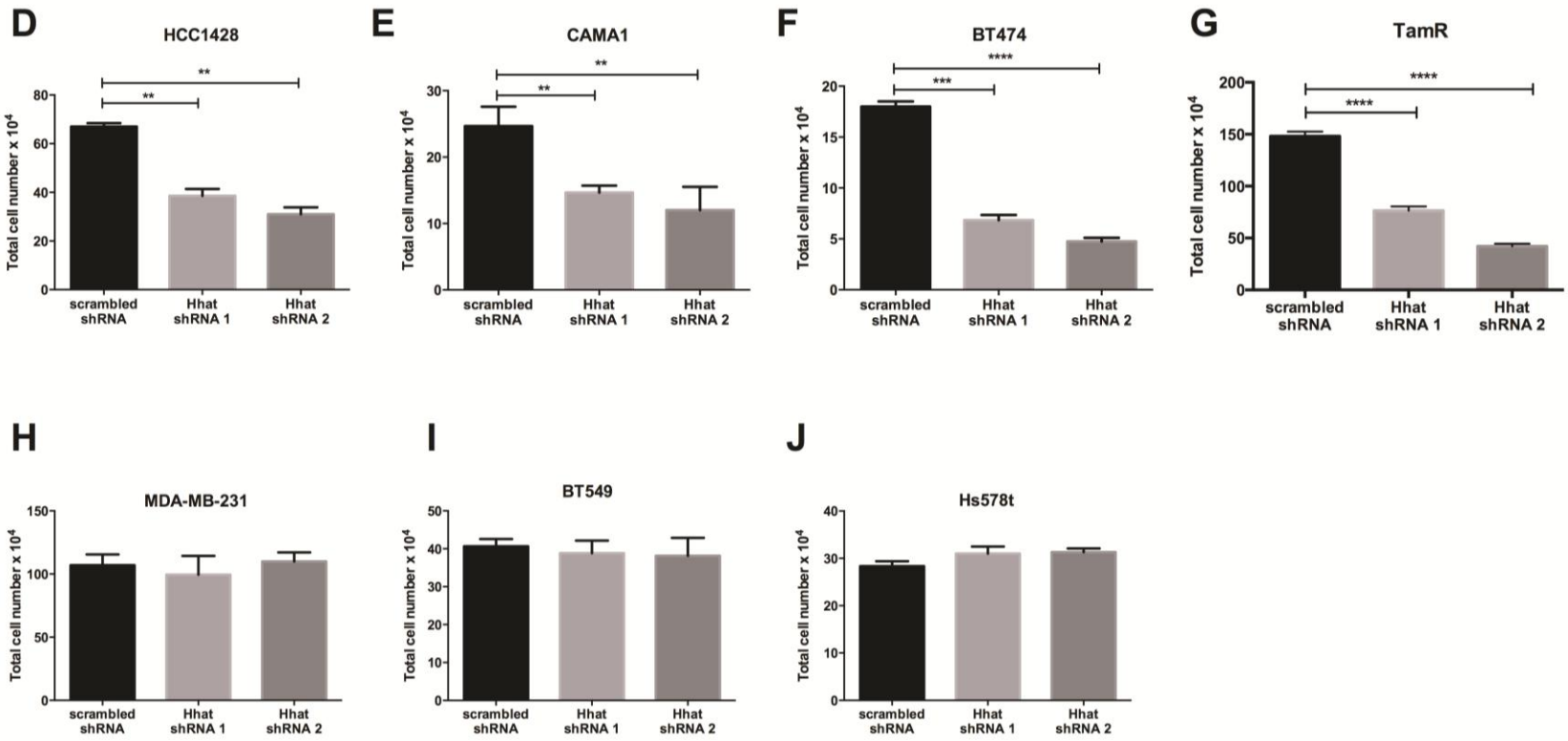

Figure 2.2 continued. Hhat depletion reduces proliferation of ER positive breast cancer cells. D-J, Total cell numbers at day 6 for (D) HCC1428, (E) CAMA-1, (F) BT474, (G) TamR, (H) MDA-MB-231, (I) BT549, and (J) Hs578t. Cells stably expressing scrambled or Hhat shRNAs were seeded at 5-7x10^4 cells/well, depending on cell type, in 6-well plates and cell numbers were quantified on day 6. Bars represent mean ± SD (n=3). Three independent experiments were performed in duplicate using cells at threedifferent passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

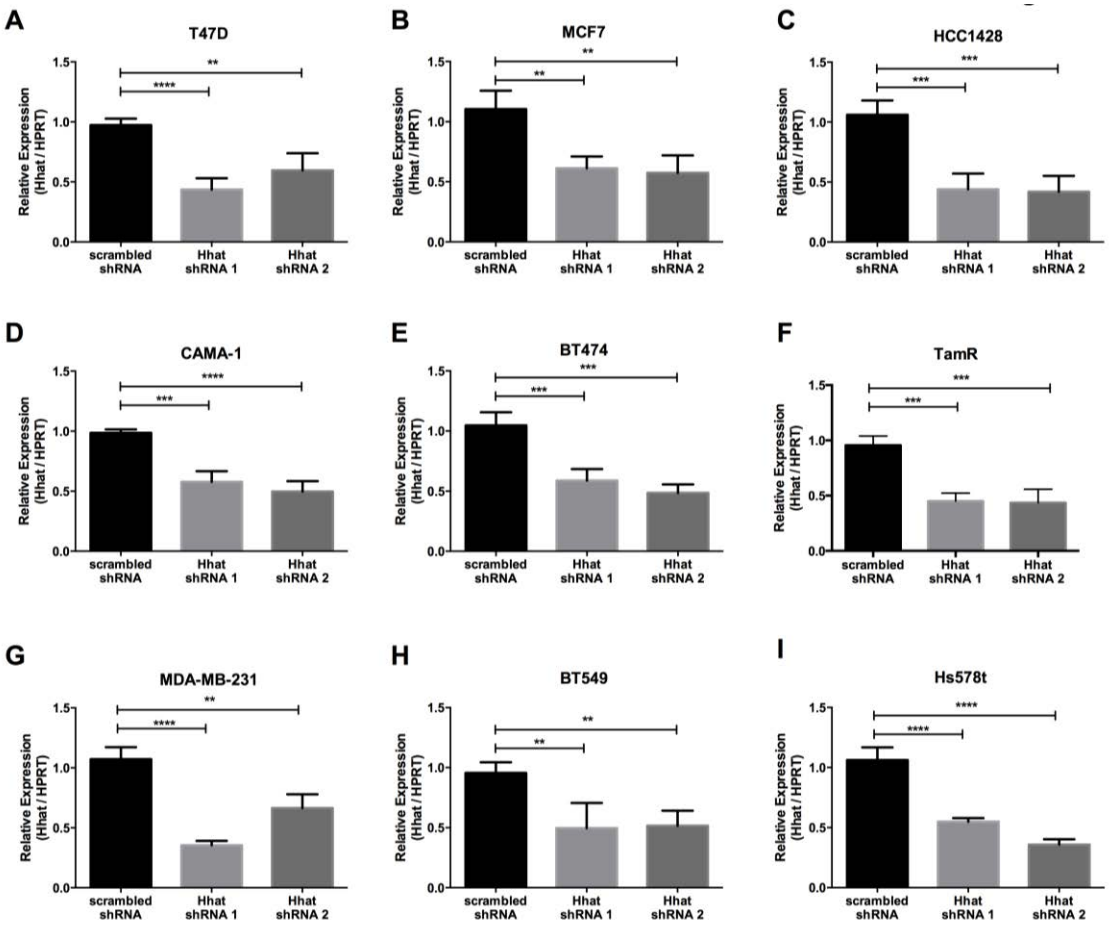

Figure 2.3 Hhat knockdown in breast cancer cells. A-I, T47D (A), MCF (B), HCC1428 (C), CAMA-1 (D), BT474 (E), TamR (F), MDA-MB-231 (G), BT549 (H), and Hs578t (I) cells were transduced with either control scrambled or two different Hhat shRNA expressing lentiviruses and selected in puromycin. qRT-PCR was performed to determine the relative expression of Hhat mRNA. Bars represent mean ± SD (n=3) for all panels. Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

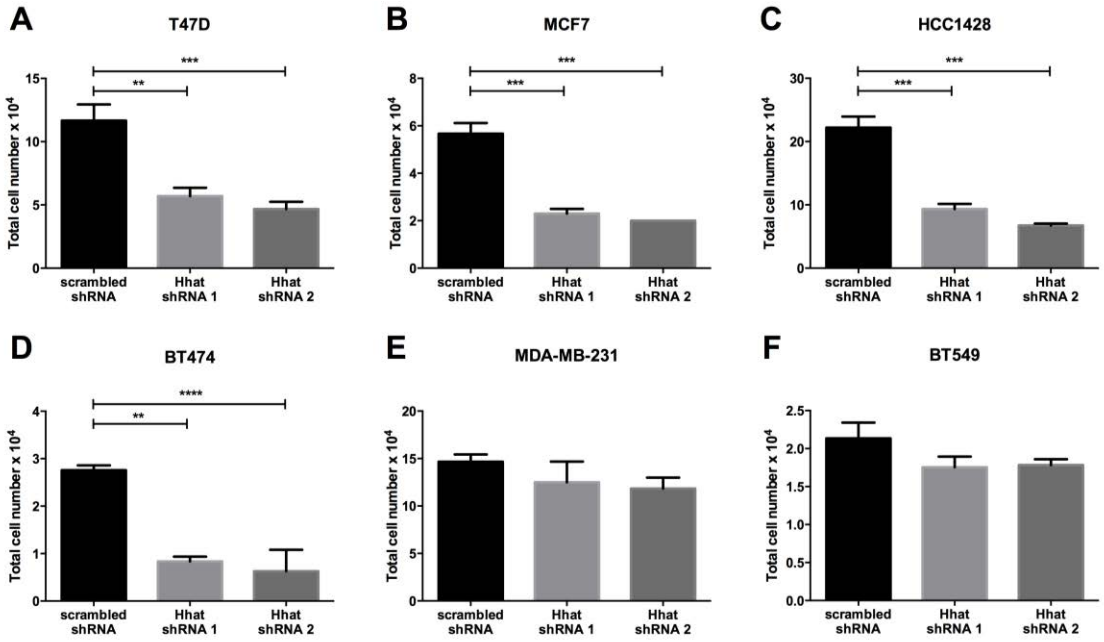

Figure 2.4 Hhat depletion reduces anchorage independent proliferation of ER positive cells. A-F, indicated breast cancer cell lines stably expressing scrambled or Hhat shRNAs were seeded at 1-2x10^4 cells/well in 24-well ultra-low adherence plates, and cell numbers were quantified 14 days later. Bars represent mean ±SD (n=3) for all panels. Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

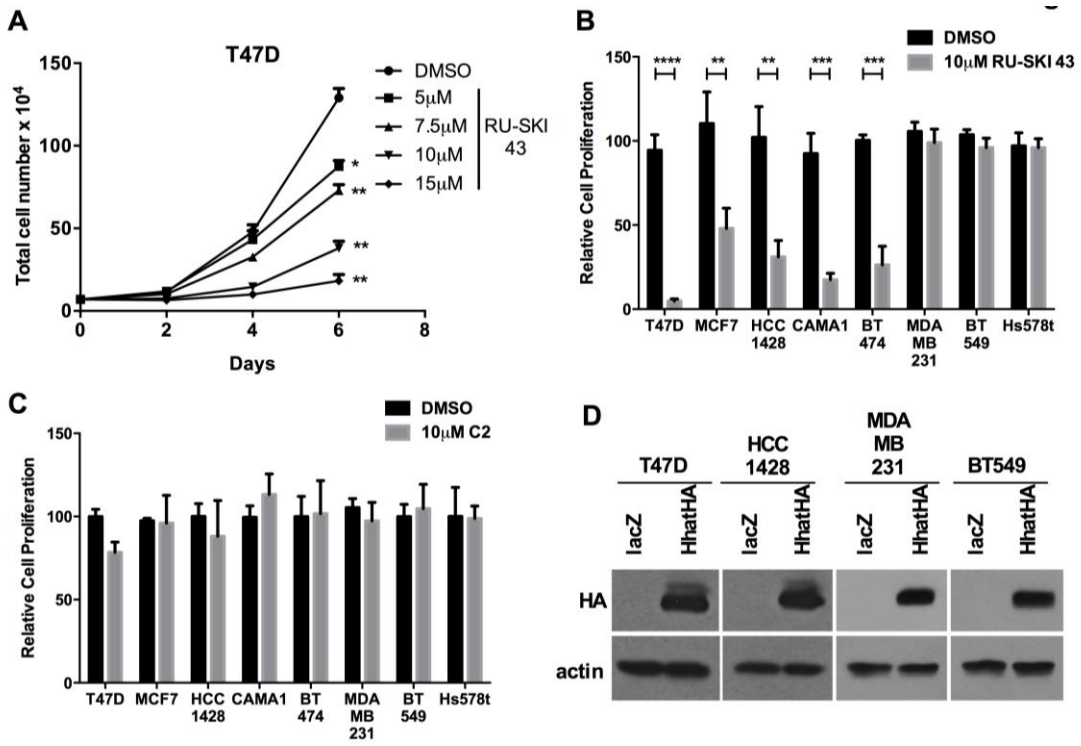

Figure 2.5 Hhat inhibition with RU-SKI 43 results in decreased proliferation of ER positive cells. A, T47D cells were seeded at $7x10^4$ cells/well in 6-well plates. 24hrs post seeding, cells were treated with either DMSO or the indicated concentrations of RU-SKI 43. Media was changed every 48hrs and cell numbers were quantified 2, 4, and 6 days post treatment. B, indicated cell lines were seeded at $5\text{-}7x10^4$ cells/well in 6-well plates. 24hrs post seeding, cells were treated with either DMSO or 10μM RU-SKI 43. Cell numbers were quantified 6 days post treatment and expressed relative to growth in DMSO (100 x (RU-SKI 43/DMSO)). C, indicated cell lines were seeded at $5\text{-}7x10^4$ cells/well in 6-well plates. 24hrs post seeding, cells were treated with either DMSO or 10μM C2. Cell numbers were quantified 6 days post treatment and expressed relative to growth in DMSO. D, cell lysates from T47D, HCC1428, MDA-MB-231, and BT549 cells stably expressing LacZ or HhatHA were analyzed directly by Western blotting.

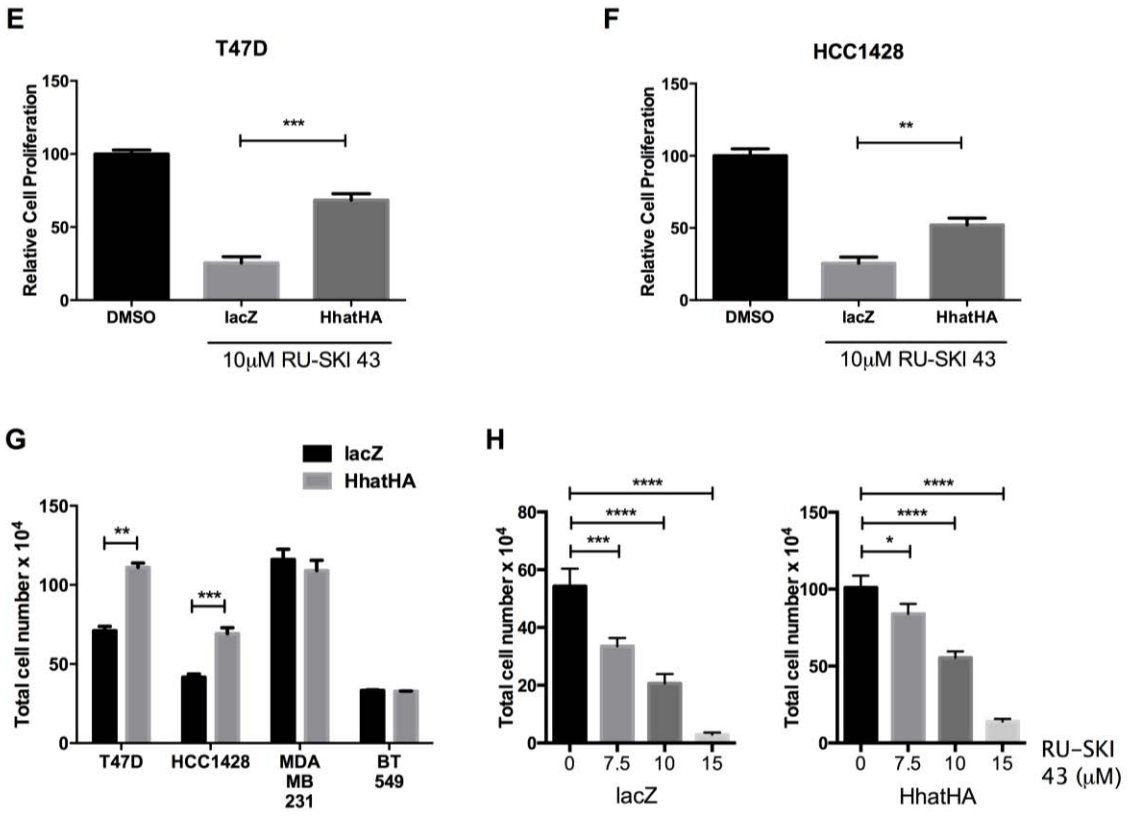

Figure 2.5 continued. Hhat inhibition with RU-SKI 43 results in decreased proliferation of ER positive cells. E-F, (E) T47D and (F) HCC1428 cells stably expressing LacZ or HhatHA were seeded at $7x10^4$cells/well in 6-well plates and grown in media containing DMSO or 10μM RU-SKI 43. Cell numbers were quantified on day 6 and expressed relative to DMSO treated cells. The increase in proliferation between Hhat and LacZ overexpressing cells in the presence of RU-SKI 43 is 176% and 106%, for T47D and HCC1428 respectively. G, growth curves for T47D, HCC1428, MDA-MB-231, and BT549 cells stably expressing LacZ or HhatHA. Cells were seeded at $5\text{-}7x10^4$ cells/well and cell numbers were quantified on day 6. The increase in proliferation in response to overexpressing Hhat in untreated cells is 56% and 61%, for T47D and HCC1428 respectively. H, T47D cells overexpressing lacZ or HhatHA were cultured in the presence of DMSO or the indicated concentrations of RU-SKI 43. Cells numbers were quantified on day 6. Bars represent mean ±SD (n=3) for all panels. Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

Hhat overexpression results in increased proliferation of ER positive cells – We next performed a gain of function experiment by testing the effect of Hhat overexpression. Stable lines of ER positive (T47D, HCC1428) and ER negative (MDA-MB-231, BT549) cells expressing either control LacZ or Hhat (Fig. 2.5D) were generated. T47D and HCC1428 cells expressing Hhat exhibited 56% and 61% increases, respectively, in cell proliferation compared to control cells expressing LacZ, while overexpression of Hhat in ER negative cells had no effect on cell proliferation (Fig. 2.5G). These findings indicate that increased Hhat activity can enhance ER positive cell proliferation. We then compared the response of cells stably expressing LacZ or Hhat to increasing concentrations of RU-SKI 43. Hhat overexpression blunted the inhibitory effect of RU-SKI 43 on cell proliferation (Fig. 2.5H), supporting the hypothesis that the effect of RU-SKI 43 is mainly due to inhibition of Hhat.

RU-SKI 43 does not alter ERα localization or activation – To examine whether Hhat functions through an ERα-dependent mechanism, we examined the effects of Hhat inhibition on ERα palmitoylation, localization, and activation. ERα has been reported to be palmitoylated, and palmitoylation has been proposed to mediate localization of a subpopulation of ERα to the plasma membrane [523, 524]. We used ^{125}I-iodopalmitate, a radioiodinated palmitate analog that allows for sensitive and robust detection of palmitoylated proteins in cells [459]. However, we were unable to detect incorporation of ^{125}I-iodopalmitate into either endogenous or overexpressed ERα in MCF7 cells. To determine whether RU-SKI 43 affects ERα localization to the plasma membrane, the subcellular localization of endogenous ERα was compared in MCF7 cells treated with

either DMSO or RU-SKI 43. ERα localized to the nucleus, cytoplasm, and plasma membrane, consistent with previous reports [523, 524], and treatment with RU-SKI 43 did not alter the ERα localization pattern (Fig. 2.6A). Finally, the ability of estradiol to induce phosphorylation of ERα at Ser118, a marker of receptor activation [525, 526], was not altered by treatment with RU-SKI 43 (Fig. 2.6B). These data indicate that the effect of the Hhat inhibitor RU-SKI 43 on ER positive cell proliferation is not due to a direct modulation of ERα localization or activation.

Non-canonical Shh signaling regulates proliferation of a subset of breast cancer cells – Hhat is the palmitoyl acyltransferase for the hedgehog family of proteins and is required for efficient Shh signaling [19, 23]. Therefore, we examined whether the effect of Hhat on proliferation is mediated through hedgehog signaling. First, we quantified the expression of hedgehog pathway components in breast cancer cells. Shh mRNA was expressed in T47D, MCF7, HCC1428, BT474, and MDA-MB-231 cells (Fig. 2.7A). Ihh was detected in MDA-MB-231 and BT549 cells, and Dhh was detected in T47D and MDA-MB-231 cells (Fig. 2.8A,B). Ptch-1 and Ptch-2 expression was detectable in nearly all cells (Figs. 2.7B, 2.8C). Although Smo was expressed in T47D, BT474, and BT549 cells (Fig. 2.7C), little to no Gli-1 or Gli-2 was expressed in these cells (Figs. 2.7D, 2.8D), suggesting either a cell non-autonomous or non-canonical role for Shh. Hs578t, which does not respond to Hhat depletion or inhibition (Figs. 2.2J, 2.5B), was the only cell line that expressed both Smo and Gli-1 (Fig. 2.7C,D). Repressors of the Shh pathway were only detected in a few cell lines (hHIP) or at very low levels (Gli-3) (Fig. 2.8E,F).

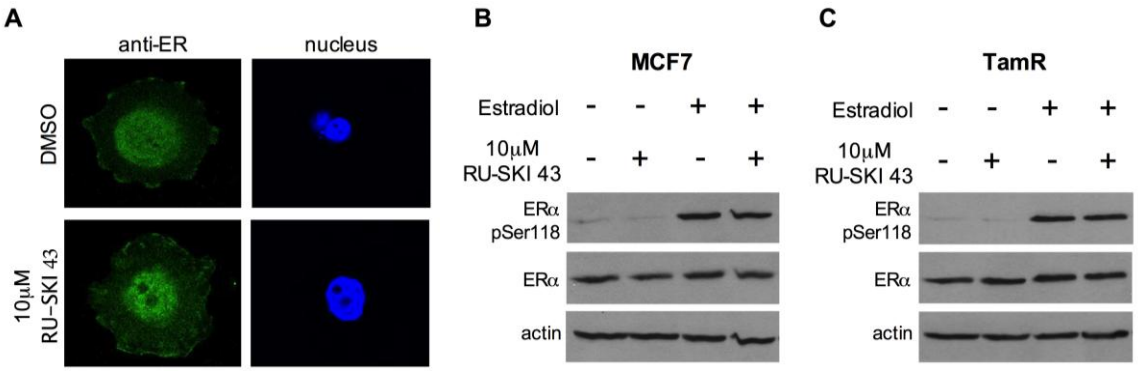

Figure 2.6 RU-SKI 43 does not alter localization or activation of ERα. A, MCF7 cells were cultured in the presence of DSMO or 10μM RU-SKI 43 for 4 h. Cells were fixed and stained with anti-ERα. Three independent experiments were performed using cells at three different passages. B-C, MCF7 (B) or TamR (C) cells were treated with DSMO or 10μM RU-SKI 43 for 4 h. Cells were treated with ethanol or 17β-estradiol for 30 minutes prior to lysis. Cell lysates were analyzed directly by Western blotting with indicated antibodies. Three independent experiments were performed in duplicate using cells at three different passages.

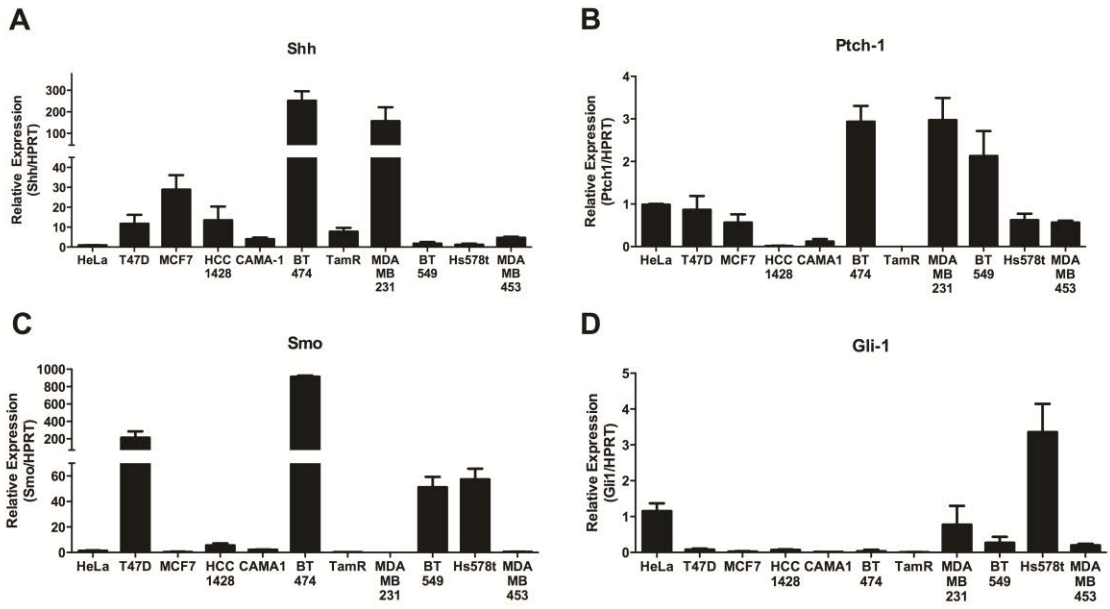

Figure 2.7 Analysis of Shh signaling pathway components in breast cancer cells. A-D, expression of (A) Shh, (B) Ptch-1, (C) Smo, and (D) Gli-1 mRNAs in indicated breast cancer cell lines and a control cervical cancer (HeLa) cell line, was measured by qRT-PCR. Expression of individual genes is shown relative to the expression in HeLa cells, which is set to 1. Bars represent mean ± SD (n=3). Experiments were performed twice in triplicate.

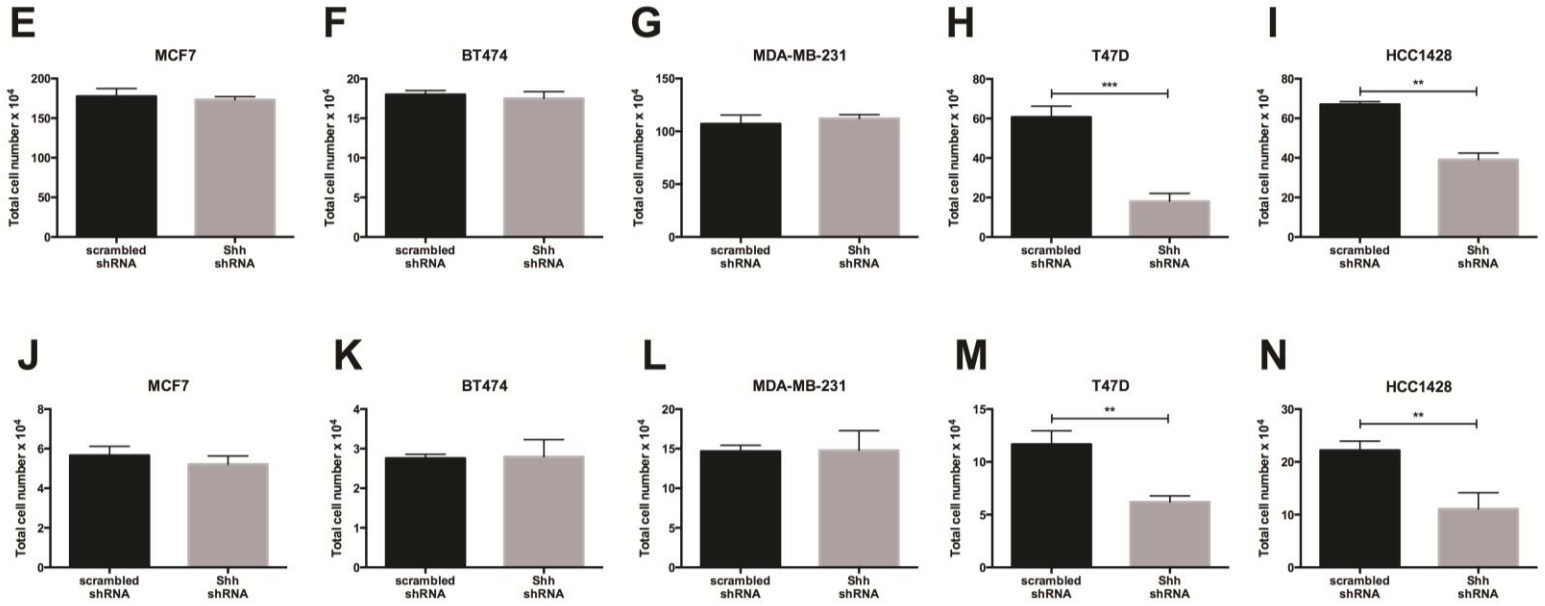

Figure 2.7 continued. Analysis of Shh signaling pathway components in breast cancer cells. E-I, indicated breast cancer cells stably expressing scrambled or Shh shRNAs were seeded at 5-7x10^4 cells/well, depending on cell type, in 6-well plates and cell numbers were quantified on day 6. J-N, indicated breast cancer cell lines stably expressing scrambled or Shh shRNAs were seeded at 1-2x10^4 cells/well in 24-well ultra-low adherence plates, and cell numbers were quantified 14 days later. For E-N, bars represent mean ±SD (n=3). Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

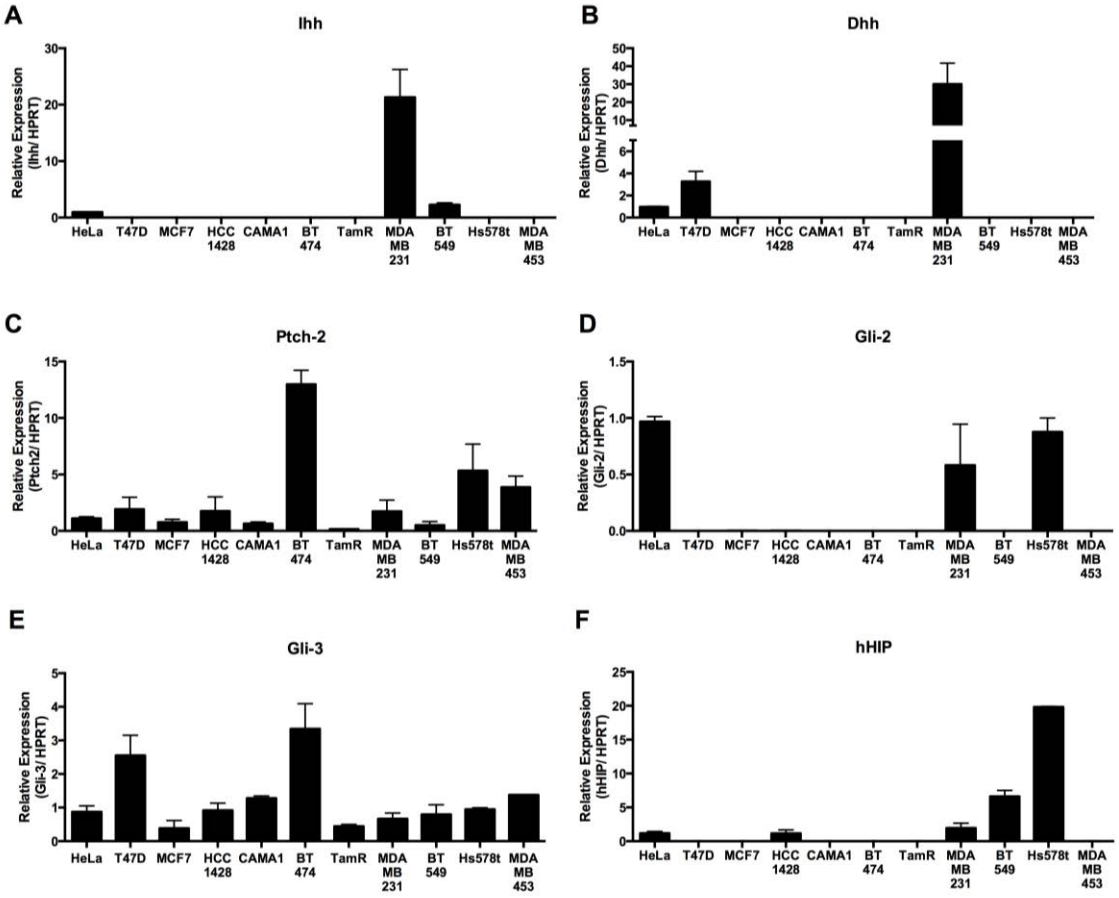

Figure 2.8 Hedehog pathway expression in breast cancer cells. A-F, expression of (A) Ihh, (B) Dhh, (C) Ptch-2, (D) Gli-2, (E) Gli-3, and (F) hHIP mRNAs in indicated breast cancer cell lines and a control cervical cancer (HeLa) cell line, was measured by qRT-PCR. Expression of individual genes is shown relative to the expression in HeLa cells, which is set to 1. Bars represent mean ± SD (n=3). Experiments were performed twice in triplicate.

Several breast cancer cell lines (T47D, MCF7, HCC1428, BT474, and MDA-MB-231) express high levels of Shh (Fig. 2.7A). To test whether the growth of these cell lines was dependent on Shh, stable expression of Shh targeting shRNAs was used to reduce Shh levels (Fig. 2.9A-E). No effect of Shh depletion was observed on either anchorage dependent or independent growth in MCF7, BT474, and MDA-MB-231 cells (Fig. 2.7E-G, J-L). To investigate whether the lack of response to Shh knockdown in these three cell lines was due to upregulation of other hedgehog ligands, levels of Ihh and Dhh were quantified in Shh depleted cells. Neither Ihh nor Dhh were detected in MCF7 and BT474 (Ct values above 35) in either scrambled control or Shh knockdown cells. In MDA-MB-231 cells, Ihh and Dhh expression was detected but did not increase after Shh knockdown (Fig. 2.9F,G). These data indicate that certain ER positive cells require Hhat but not Shh for proliferation, suggesting a Shh independent role for Hhat.

We identified two cell lines, T47D and HCC1428, in which Shh depletion reduced both anchorage-dependent (Fig. 2.7H,I) and anchorage-independent proliferation (Fig. 2.7M,N). We next asked whether decreased Shh signaling was responsible for the reduction in cell proliferation observed upon Hhat inhibition. If RU-SKI 43 reduces cell proliferation through Shh, then addition of exogenous, recombinant Shh(C24II) should rescue the growth of these cells in the presence of RU-SKI 43. When Shh(C24II) was added to T47D cells, no effect on cell proliferation was observed (Fig. 2.10A). However, we and others have previously shown that in Shh producing cells, the hedgehog signaling machinery is saturated and a response to exogenous Shh is only revealed after endogenous Shh depletion [24, 345, 527]. Addition of Shh(C24II) rescued, in part, thegrowth defect of Shh-depleted T47D cells, but had no effect on T47D cells expressing

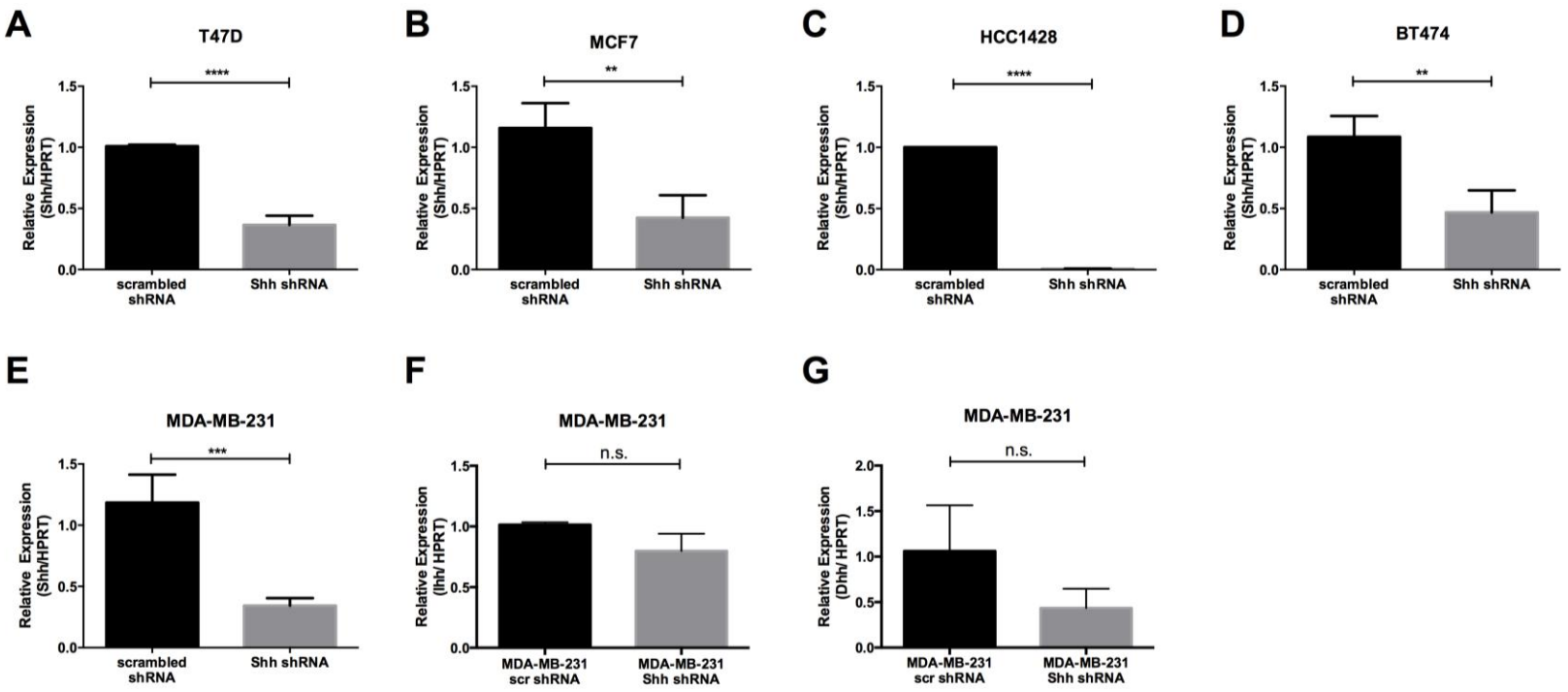

Figure 2.9 Shh knockdown in breast cancer cells. A-E, T47D (A), MCF (B), HCC1428 (C), BT474 (D), and MDA-MB-231 (E) cells were transduced with either control scrambled or Shh shRNA expressing lentiviruses and selected in puromycin. qRT-PCR was performed to determine the fold change in Shh expression. F-J, MDA-MB-231 cells were transduced with either control scrambled or Shh shRNA expressing lentiviruses and selected in puromycin. qRT-PCR was performed to determine the fold change in Ihh (F) and Dhh (G) expression. For B, D, E Bars represent mean ± SD (n=3). Experiments were performed three times in triplicate. For panels B, D, and E Bars represent mean ± SD (n=3). Three independent experiments were performed in triplicate using cells at three different passages. For panels A, C, F, and G, bars represent mean ± SD (n=2). Two independent experiments were performed in triplicate using cells at two different passages. *P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0001; Student's t test.

the scrambled control shRNA (Fig. 2.10B). However, treatment of Shh-depleted cells with RU-SKI 43 further decreased their growth, suggesting a role for Hhat in addition to Shh signaling (Fig. 2.10B).

To examine whether canonical Smo-mediated Shh signaling is required for the proliferation of these cells, the effect of LDE-225, a Smo inhibitor, was analyzed. Nanomolar concentrations of LDE-225 inhibit canonical Shh signaling [528] and decrease the growth of LDE-225 sensitive tumor cells [529]. We used LDE-225 at 0.1μM, a concentration 100x higher than IC_{50} for binding of LDE-225 to Smo [530], and found it had no effect on the proliferation of any of the breast cancer cell lines (Fig. 2.10C), suggesting that Smo-mediated signaling is absent in these cells. This is consistent with our finding that T47D and HCC1428 cells have little to no Gli-1 expression (Fig. 2.7D).

Hhat depletion or inhibition reduces proliferation of HER2 amplified cells – ER positive/HER2 positive BT474 cells are sensitive to Hhat depletion or inhibition (Figs. 2.3-2.5, Fig. 2.11A). We therefore tested whether Hhat activity is also required for the growth of HER2 positive cells that are ER negative. Treatment of MDA-MB-453 and SK-BR-3 cells with RU-SKI 43 reduced proliferation, while C2 had no effect (Fig. 2.11B,C, Fig. 2.12A). Depletion of Hhat in MDA-MB-453 cells (Fig. 2.12B) also led to a significant reduction in proliferation (Fig. 2.12C). Thus, Hhat activity is required for the proliferation of HER2 amplified cells independently of ER status. Furthermore, inhibition of both Hhat and HER2 by combined treatment with RU-SKI 43 and lapatinib resulted in

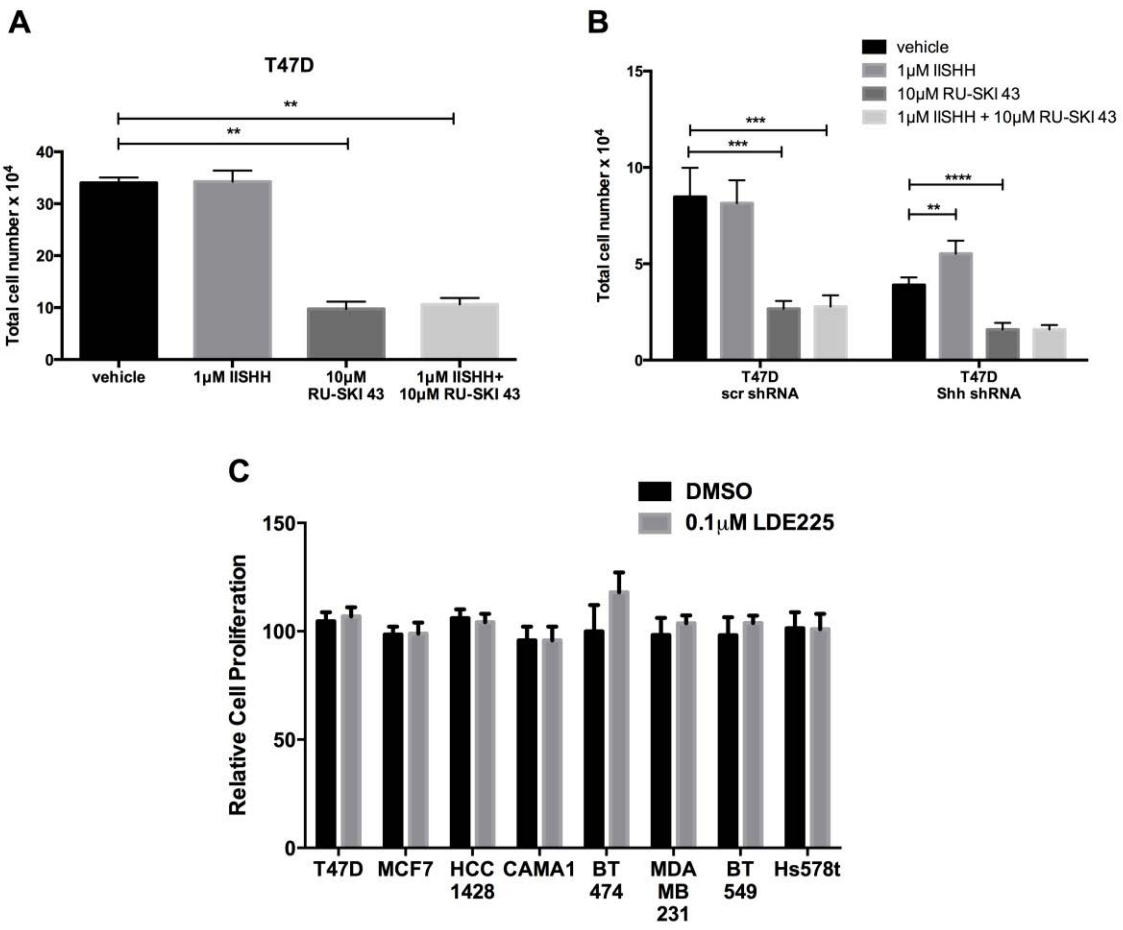

Figure 2.10 Evidence for non-canonical Shh signaling in breast cancer cells. A, T47D cells were cultured in the presence of 1μM Shh(CII24), 10μM RU-SKI 43, or both for 6 days. Cell numbers were quantified and normalized to vehicle treated cells (100 x (drug/vehicle)). Bars represent mean ± SD (n=3). Experiments were performed twice in triplicate. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test. B, T47D cells were transduced with either a control scrambled or Shh shRNA expressing lentivirus and selected in puromycin. Cells were then cultured in the presence of 1μM Shh(CII24), 10μM RU-SKI 43, or both for 6 days. Cell numbers were quantified and normalized to vehicle treated cells (100 x (drug/vehicle)). Bars represent mean ± SD (n=3). Experiments were performed twice in triplicate. ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test. C, indicated cell lines were seeded at 5-7x10^4 cells/well in 6-well plates. 24hrs post seeding, cells were treated with either DMSO or 0.1μM LDE225. Cell numbers were quantified 6 days post treatment and expressed relative to growth in DMSO. Bars represent mean ±SD (n=3). Three independent experiments were performed in triplicate using cells at three different passages.

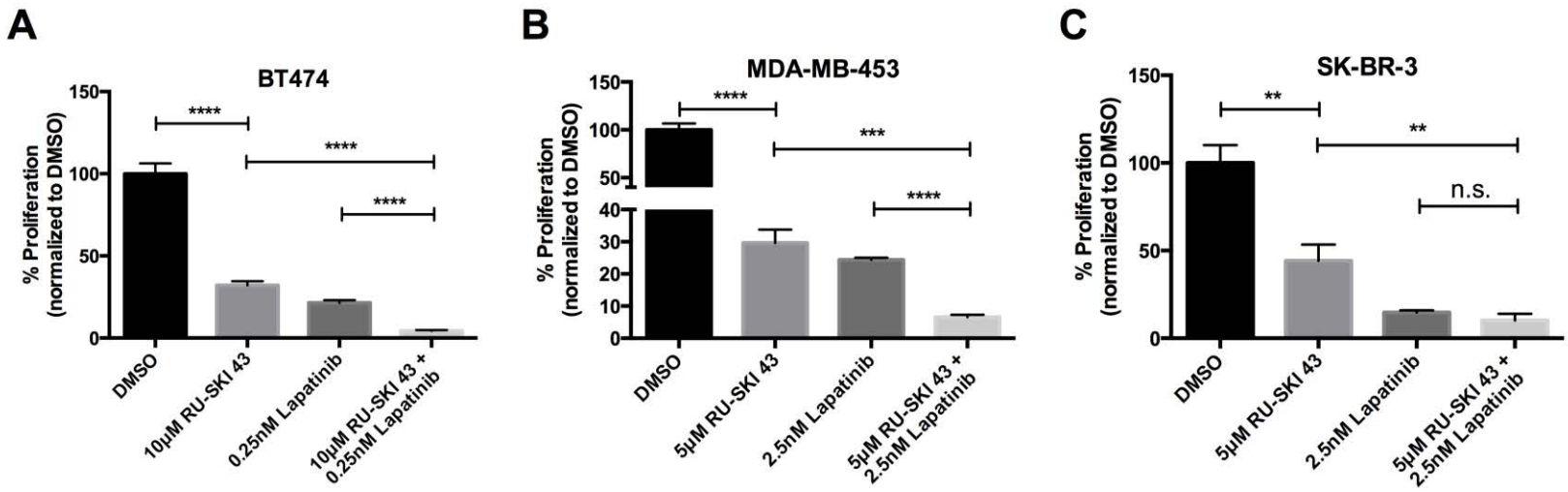

Figure 2.11 Hhat inhibition reduces proliferation of HER2 amplified cells. A-C, BT474 (A), MDA-MB-453 (B), and SK-BR3 (C) cells were cultured for 6 days in the presence of DMSO, RU-SKI 43 alone or in combination with indicated concentrations of lapatinib. Cell numbers were quantified and normalized to vehicle treated cells (100 x (drug/vehicle). Bars represent mean ±SD (n=3) for all panels. Each experiment was performed using three separate passages of cells in triplicate. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

significantly reduced proliferation of BT474 and MDA-MB-453 cells when compared to treatment with either agent alone (Fig. 2.11A,B). Taken together, these data suggest that Hhat inhibition may be combined with current HER2 targeted therapies to achieve a more potent inhibition of breast cancer cell proliferation.

Combined inhibition of Hhat and PI3K/mTOR effectively reduces breast cancer cell proliferation – Activation of PI3K/mTOR signaling occurs in up to a quarter of both ER positive and HER2 positive breast cancers [404] and several inhibitors are currently in clinical trials [531]. Furthermore, increased signaling through this pathway is also associated with resistance to available therapies [404]. Therefore, we next examined whether RU-SKI 43 could be effectively combined with PI3K or mTOR inhibitors to reduce cell proliferation. Combined treatment of ER positive breast cancer cells with RU-SKI 43 and either the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin resulted in a further decrease in cell proliferation compared to either drug alone (Fig. 2.13A-C). Thus, simultaneous inhibition of Hhat and PI3K/mTOR signaling effectively reduces breast cancer cell proliferation.

Hhat depletion or inhibition reduces proliferation of tamoxifen resistant cells – Tamoxifen is the most widely used hormone therapy for breast cancer [397]. We therefore investigated whether RU-SKI 43 could enhance the ability of tamoxifen to reduce ER positive cell proliferation. Combined treatment with RU-SKI 43 and 4-hydroxytamoxifen (4-OH Tam) significantly reduced proliferation in T47D, HCC1428, and MCF7 cells compared to either drug alone (Fig. 2.14A-C). We then tested whether

tamoxifen resistant cells retained sensitivity to Hhat inhibition. BT474 cells are tamoxifen resistant (Fig. 2.14D) due to HER2 amplification, but exhibited reduced proliferation after Hhat knockdown (Figs. 2.2F, 2.4D) or inhibition (Figs. 2.5B, 2.11A, 2.14D). We next examined the effect of Hhat inhibition in cells that acquire tamoxifen resistance in the absence of HER2 amplification. We used a tamoxifen resistant clone, TamR, generated by culturing MCF7 cells in the presence of 10^{-7}M 4-OH Tam [532], and verified that this clone does not have HER2 amplification (Fig. 2.1). Depletion of Hhat in TamR cells (Fig. 2.3F) significantly decreased cell proliferation (Fig. 2.2G). In addition, treatment of TamR cells with RU-SKI 43 reduced cell proliferation by 60%, similar to the effect observed in MCF7 cells (Fig. 2.14C,E). ER activation in TamR cells was not altered in the presence of RU-SKI 43 (Fig. 2.6C). Furthermore, the combination of RU-SKI 43 and tamoxifen led to a more potent inhibition of TamR proliferation (Fig. 2.14E) compared to RU-SKI 43 treatment alone. Taken together, these data suggest that Hhat can serve as a target in cells that acquire tamoxifen resistance through either HER2 amplification or other mechanisms.

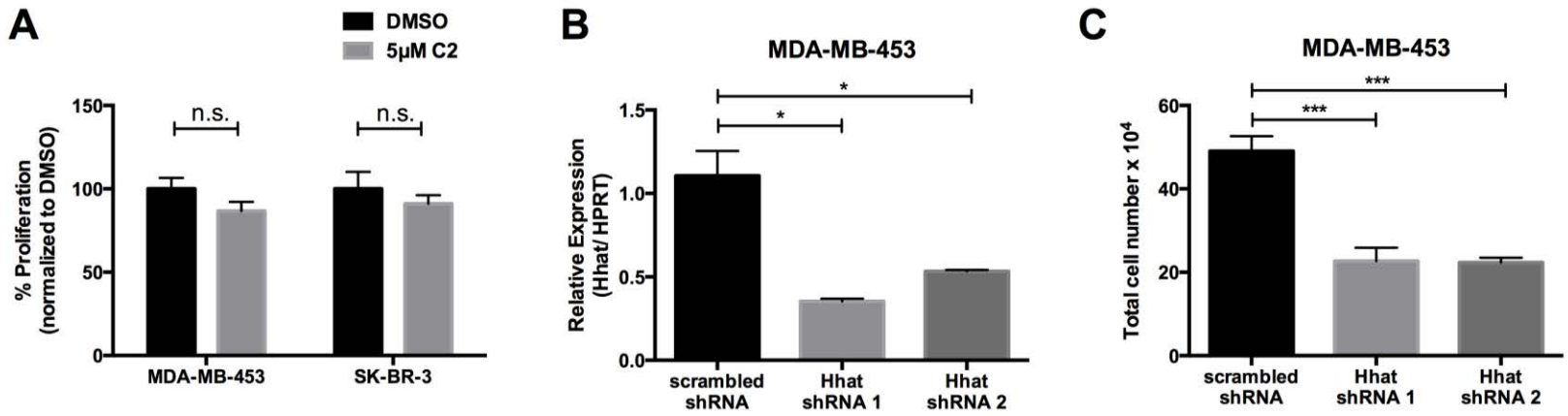

Figure 2.12 Hhat depletion reduces proliferation of MDA-MB-453 cells. A, MDA-MB-453 and SK-BR-3 cells were cultured for 6 days in the presence of DMSO or 5μM C2. Cell numbers were quantified and normalized to DMSO treated cells (100 x (drug/DMSO). Bars represent mean ±SD (n=3). Three independent experiments were performed in duplicate using cells at three different passages. B, MDA-MB-453 cells were transduced with either control scrambled or two different Hhat shRNA expressing lentiviruses and selected in puromycin. qRT-PCR was performed to determine the fold change in Hhat expression. Bars represent mean ± SD (n=3). Three independent experiments were performed in duplicate using cells at three different passages. C, MDA-MB-453 cells stably expressing scrambled or Hhat shRNAs were seeded at $7x10^4$ cells/well in 6-well plates and cell numbers were quantified on day 6. Bars represent mean ± SD (n=3). Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

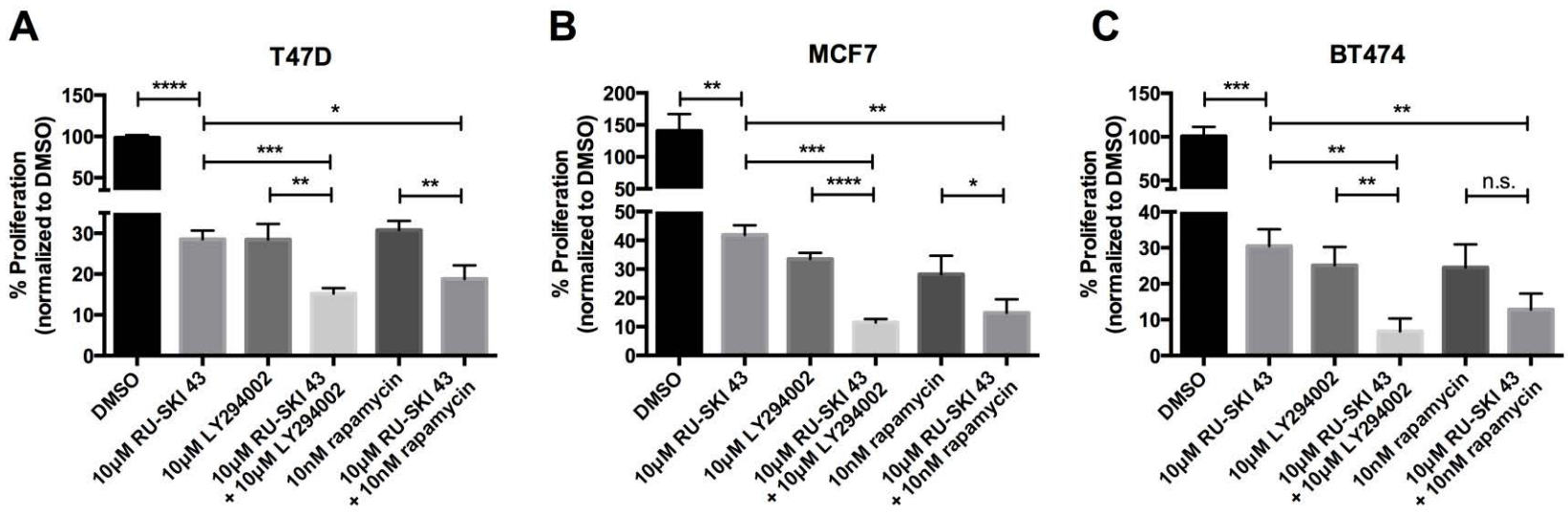

Figure 2.13 Combined inhibition of Hhat and PI3K/mTOR effectively reduces breast cancer cell proliferation. A-C, T47D (A), MCF7 (B), and BT474 (C) cells were cultured for 6 days in the presence of 10μM RU-SKI 43 alone or in combination with 10μM LY294002 or 10nM rapamycin. Cell numbers were quantified and normalized to DMSO treated cells (100 x (drug/DMSO)). Bars represent mean ±SD (n=3) for all panels. Three independent experiments were performed in duplicate using cells at three different passages. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

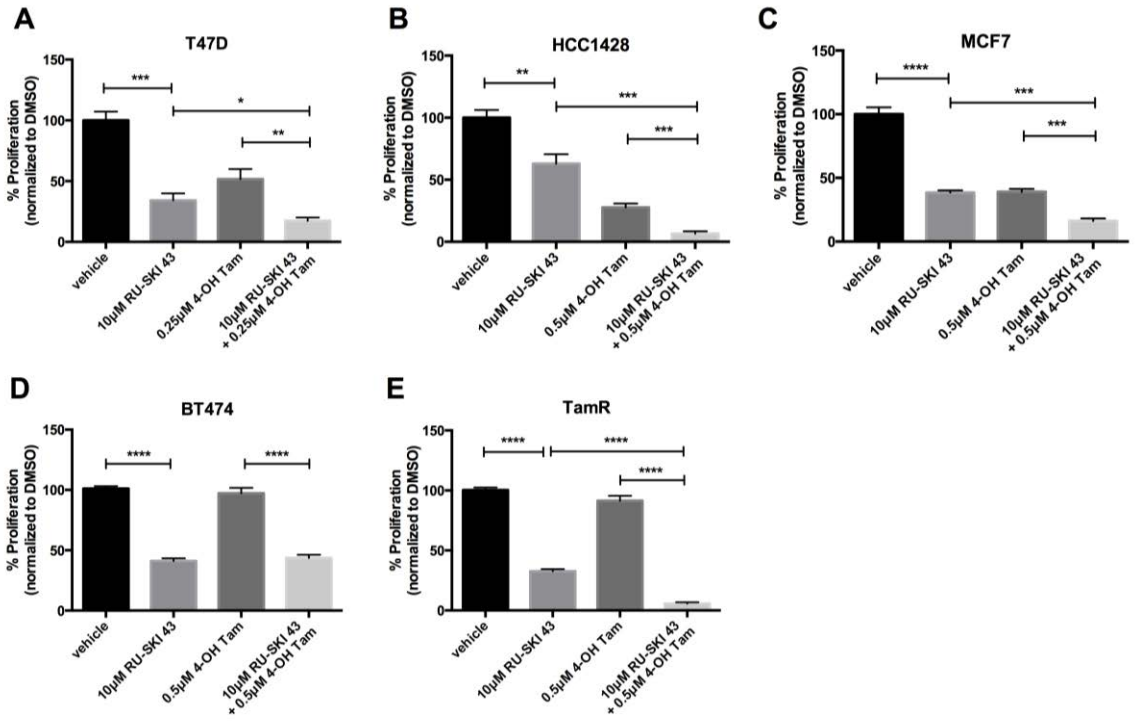

Figure 2.14 Tamoxifen resistant cells are sensitive to Hhat inhibition. A-D, T47D (A), HCC1428 (B), MCF7 (C), BT474 (D), and TamR (E) cells were cultured for 6 days in the presence of vehicle, 10μM RU-SKI 43 alone or in combination with indicated concentrations of 4-hydroxytamoxifen (4-OH Tam). Cell numbers were quantified and normalized to vehicle treated cells (100 x (drug/vehicle). Bars represent mean ± SD (n=3) for all panels. Three independent experiments were performed in duplicate using cells at three different passage. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; Student's *t* test.

Discussion

In this study, we used genetic and pharmacologic methods to establish Hhat as a critical regulator of breast cancer cell growth. Hhat depletion or treatment with the selective Hhat inhibitor RU-SKI 43 reduced both anchorage-dependent and anchorage-independent proliferation of ER positive cells (Figs. 2.2, 2.4). Hhat knockdown or inhibition also reduced the growth of HER2 positive and tamoxifen resistant cells (Figs. 2.2, 2.11, 2.14). Inhibition of breast cancer cell growth by RU-SKI 43 was dose dependent and was rescued by Hhat overexpression (Fig. 2.5). Treatment with C2, a compound that is structurally similar to RU-SKI 43 but does not inhibit Hhat activity [459], had no effect on proliferation (Fig. 2.5). We have previously demonstrated that the inhibitory effect of RU-SKI 43 is selective for Hhat, as this compound does not inhibit palmitoylation of H-Ras and Fyn, myristoylation of c-Src, or fatty acylation of Wnt3a by Porcupine, another member of the MBOAT family [459]. Overexpressing increasing amounts of Hhat, but not Porcupine, decreases the inhibitory effect of RU-SKI 43 on Shh palmitoylation [459]. Moreover, overexpression of Hhat reduced the inhibitory effect of RU-SKI 43 on breast cancer cell proliferation (Fig. 2.5H). It is possible that breast tumors that overexpress Hhat due to gene amplification might require higher doses of Hhat inhibitor. However, our finding that RU-SKI 43 inhibits the growth of T47D cells, which express relatively high levels of Hhat compared to other cell lines (Fig. 2.2A), suggests that Hhat inhibition is a viable approach to reducing breast cancer cell growth. Taken together, these data suggest that the primary target of RU-SKI 43 is Hhat, and provide the first identification of Hhat as a novel target in breast cancer.

Hhat was identified as the palmitoyl acyltransferase for Shh and the hedgehog family of proteins [19, 23], and Hhat inhibition has been shown to block Shh signaling [459]. Thus, it was important to monitor expression of Shh and hedgehog signaling pathway components in breast cancer cells. There is general agreement between the findings reported here and in four other studies [182, 435, 436, 521] that examined expression levels of Shh pathway components in four of the same cell lines (T47D, MCF7, MDA-MB-231, and BT474) that we analyzed: 1) Shh is expressed in MCF7, T47D, and MDA-MB-2312) Ptch-1 and 2 are expressed in all four cell lines, and 3) Smo is expressed in T47D and BT474 but not in MCF7 and MDA-MB-231 cells. However, in contrast to other studies, we did not detect Ihh, Dhh, Gli-1 or Gli-2 expression in MCF7 or T47D cells (Figs. 2.7, 2.9). Differences in Gli expression among the four studies may be due to differences in culture methods or confluence state of cells.

Our study addresses two key questions regarding the role of Shh in breast cancer: 1) Do Shh expressing cells exhibit an autocrine response to Shh? 2) If so, does this occur through canonical or non-canonical signaling? Here, we identify two cell lines, T47D and HCC1428, where knockdown of Shh reduced anchorage dependent and independent proliferation (Fig. 2.7). T47D cells can also undergo increased proliferation in response to exogenous Shh, but this increase is only evident after endogenous levels of Shh are depleted (Fig. 2.10). However, T47D and HCC1428 cells neither express Gli-1 (Fig. 2.7) nor respond to treatment with the Smo inhibitor LDE-225 (Fig. 2.10), indicating the presence of non-canonical Shh signaling. Others have also noted that treatment with cyclopamine, a Smo inhibitor, reduces proliferation of certain breast cancer cells, but that this does not correlate with Smo expression [435] or inhibition [521]. In this study, we

used LDE-225 at 0.1μM, a concentration 100x higher than IC_{50} for binding of LDE-225 to Smo [530], and found no effect on proliferation of any of the breast cancer cells (Fig. 2.10). Taken together, these findings suggest that in breast cancer cells, canonical Smo mediated signaling is not operative, and cells that respond to Shh do so via non-canonical, Smo-independent signaling. This conclusion is supported by multiple recent studies documenting the existence of non-canonical, Smo-independent Shh signaling pathways in normal and cancer cells [24, 169, 173, 182, 533].

The findings presented here indicate that Hhat has regulatory roles in addition to Shh signaling. Shh depleted cells were still sensitive to Hhat inhibition and this growth defect was not rescued by addition of exogenous Shh (Fig. 2.10). Moreover, we demonstrate a requirement for Hhat, but not Shh, for proliferation of multiple ER positive cells (Figs. 2.2, 2.4, 2.7), consistent with our recent report showing that Hhat can have Shh-independent functions in pancreatic cancer cells [24]. We speculate that Hhat has substrates in addition to the hedgehog family. Studies in flies have shown that the EGF-like ligand Spitz is a substrate for Rasp, the *Drosophila melanogaster* ortholog of Hhat [259]. Although no Spitz ortholog has been identified in mammals, and none of the mammalian EGF family ligands appear to be palmitoylated by Hhat, our findings of hedgehog-independent roles of Hhat suggest that other substrates exist. We conclude that Hhat can promote breast cancer cell growth in a Shh independent manner.

All ER positive cell lines that we tested responded to Hhat depletion or inhibition by exhibiting decreased proliferation, while triple negative cell lines did not. Multiple lines of evidence argue against the possibility that Hhat operates via a direct, ER-dependent mechanism. First, despite reports that ERα is palmitoylated, ERα is unlikely to

be a direct substrate for Hhat. The active site of Hhat is oriented towards the lumen of the endoplasmic reticulum. Hhat mediated palmitoylation occurs in the ER lumen and Hhat only palmitoylates secreted proteins [19, 259]. In contrast, ERα is localized to the nucleus, cytosol and plasma membrane, and palmitoylation of ERα is thought to occur in the cytoplasm [524]. Thus, Hhat could not topologically access ERα as a substrate as ERα does not enter the secretory pathway. Second, using ^{125}I-iodopalmitate, a sensitive and robust probe for palmitoylated proteins, we were unable to detect incorporation of ^{125}I-iodopalmitate into either endogenous or overexpressed ERα. Third, RU-SKI 43 treatment did not alter the localization or activation of ERα, suggesting RU-SKI 43 does not directly affect ERα function (Fig. 2.6). Fourth, depletion or inhibition of Hhat can also inhibit the growth of HER2 positive cells that are ER negative (Fig. 2.11, 2.12B), indicating that, in the context of HER2 amplification, Hhat can modulate cell proliferation independently of ER status.

Increased PI3K/mTOR signaling occurs in up to a quarter of breast cancers [404] and upregulation of Akt signaling is associated with resistance to both endocrine and HER2 targeted therapies [404, 520]. We observed that simultaneous inhibition of PI3K/mTOR and Hhat led to a greater decrease in cell proliferation than with either agent alone (Fig. 2.13). Similarly, combined treatment with the Hhat inhibitor and tamoxifen was more effective than either drug alone (Fig. 2.14). In addition, we noted that tamoxifen resistant cells, either through HER2 amplification (BT474) or other mechanisms (TamR), maintained sensitivity to Hhat knockdown or inhibition (Figs. 2.2, 2.14). Of note, combined treatment of the TamR cells with the Hhat inhibitor and tamoxifen was more effective than RU-SKI 43 alone (Fig. 2.14). Since RU-SKI 43 did

not alter ERα activation in TamR cells (Fig. 2.6C), it is possible that other pathways induced during selection for tamoxifen resistance may contribute to the increased sensitivity in this clone. As with all pharmacologic approaches, we cannot exclude the possibility that off-target effects of RU-SKI 43, yet to be identified, contribute to the response in TamR cells. Taken together, these data underscore the therapeutic potential of using Hhat inhibitors alone or in combination with Akt/mTOR inhibitors or ER modulators to treat breast cancer and circumvent or delay resistance to current treatments.

Acknowledgements

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Footnotes

Chapter Three

Membrane Topology of Hedgehog Acyltransferase

Hedgehog acyltransferase (Hhat) catalyzes the covalent attachment of the 16-carbon fatty acid palmitate to the N-terminal cysteine of Sonic Hedgehog (Shh) [19], a modification that is critical for signaling activity [17, 20-23]. Shh signaling is required for proper embryogenesis and tissue development, and defects in Shh signaling result in abnormal development of the neural tube, gastrointestinal tract, and limbs [11]. In adults, aberrant Shh signaling promotes the initiation and progression of various tumors including gastrointestinal, pancreatic, and prostate cancers [534]. In addition, Shh signaling plays a role in maintaining cancer stem cells and in mediating resistance to cancer therapies [535]. Furthermore, recent studies elucidated the importance of Hhat activity in mediating the proliferation of pancreatic and lung squamous cell carcinomas

[24-27]. These studies established Hhat as an attractive drug target, and small molecule inhibitors of Hhat are currently under development [459].

Hhat is a multipass transmembrane protein that belongs to the membrane bound O-acyltransferase (MBOAT) family [276]. The MBOAT family is characterized by a conserved homology domain with an invariant His residue (His379 in Hhat) surrounded by hydrophobic residues and a highly conserved Asp/Asn residue (Asp339 in Hhat) surrounded by moderately hydrophobic residues. Most MBOAT enzymes transfer fatty acids and other lipids onto hydroxyl groups of membrane bound lipids. Hhat and two other MBOAT members (Porcupine and ghrelin O-acyltransferase) are unique in that they catalyze the transfer of fatty acids onto secreted proteins. Porcupine (Porcn) transfers a monounsaturated 16-carbon fatty acid onto the Wnt family of ligands [289], and ghrelin O-acyltransferase (GOAT) transfers an 8-carbon fatty acid onto the appetite stimulating peptide hormone ghrelin [279, 306].

Hhat, Porcn, and GOAT are localized in the endoplasmic reticulum (ER) and acylate proteins that travel through the secretory pathway. Since entry into the secretory pathway is necessary for Hhat mediated palmitoylation of Shh and GOAT mediated octanoylation of ghrelin [19, 282], the active site of these enzymes is most likely located on the lumenal face of the ER. Knowledge of the topological organization of MBOAT transmembrane helices would considerably enhance our understanding of the mechanism of MBOAT-mediated protein fatty acylation. A recent study of the topology of GOAT identified 11 transmembrane domains (TMDs) and one re-entrant loop, with the invariant His residue in the lumen and the highly conserved Asn residue in the cytoplasm [282]. To date, the number and orientation of TMDs within Hhat remains unknown.

To further our understanding of Hhat structure and function, we set out to determine the membrane topology of Hhat. Here, we combine *in silico* and empirical methods to experimentally determine the topological organization of Hhat across the membrane bilayer. Selective membrane permeabilization coupled with immunofluorescence and an *in vitro* protease protection assay were used to establish the presence of 10 TMDs and two re-entrant loops within Hhat. The topological organization of Hhat provides a framework for understanding its mechanism of action and may aid in the further design of Hhat inhibitors.

Experimental Procedures

Reagents and Antibodies – Reagents were purchased from the following vendors: Trypsin, digitonin, cycloheximide, chloramphenicol, Triton X-100, and anti-Flag (Sigma, St. Louis, MO); anti-Shh, anti-Myc, and anti-caveolin antibodies (Santa Cruz Biotechnology, Dallas, Texas); anti-HA (Roche, Basel, Switzerland); anti-PDI (Enzo Life Sciences, Farmingdale, NY); octylglucoside (EMD Millipore, Billerica, MA); [^{125}I] NaI (Perkin Elmer, Waltham, MA).

Mammalian Expression Plasmids – The plasmid encoding HA-tagged Hhat was generated as previously described [19]. Hhat constructs with C-terminal Flag and Myc epitope tags as well as Flag and HA epitope insertions were generated using site directed mutagenesis via the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing.

Cell Culture and Transfections – COS-1 and COS-7 cells were grown in Dulbecco's Modified Eagle's (DMEM) medium supplemented with 10% fetal bovine serum, 1mM GlutaMAX (Invitrogen, Carlsbad, CA), 50 units/ml penicillin, and 50 μg/ml streptomycin. 293FT cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50μg/ml streptomycin, 500 μg/ml Geneticin, 1mM GlutaMAX, 1mM sodium pyruvate, and 0.1 mM nonessential amino acids. Transfections were carried out using Lipofectamine 2000® (Invitrogen).

Selective Permeabilization and Indirect Immunoflourescence – COS-1 cells were transfected with indicated Hhat constructs. 24 h post transfection, cells were split onto coverslips in 6-well plates and cultured for an additional 24 h. Cells were fixed and permeabilized as previously described [282] with a few changes. Briefly, to selectively permeabilize the plasma membrane, cells were incubated with 65 μg/mL digitonin in KHM (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate) for 10 min on ice, and fixed with 3% paraformaldehyde for 10 min at room temperature. To permeabilize all cellular membranes, cells were fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Cells were incubated with indicated primary antibodies and with secondary antibodies (Alexa Flour® 488-conjugated anti-mouse IgG and Alexa Flour® 594-conjugated anti-rabbit IgG) for 45 min each. Slides were mounted with ProLong® Gold Antifade (Invitrogen). Images were collected using a Leica SP5 confocal microscope and analyzed with the Leica Application Suite software.

Protease Protection Assays – P100 membranes were prepared as previously described [19]. Briefly, 293FT cells transfected with the indicated Hhat constructs were washed with ice cold STE (100 mM NaCl, 10 mM Tris, and 1 mM EDTA (pH 7.4)), collected and centrifuged for 10 min at 1000 × g at 4°C. Cell pellets were resuspended in hypotonic lysis buffer (10 mM HEPES (pH 7.3) and 0.2 mM $MgCl_2$) and incubated on ice for 10 min, followed by dounce homogenization with 30 strokes. The homogenate was supplemented with 1.25 M sucrose and centrifuged for 45 min at 100,000 × g at 4°C. The pellets were resuspended in hypotonic lysis buffer supplemented with protease inhibitors and flash frozen. For each protease protection assay, 50 μg total membrane protein was incubated at 30°C for 30 min with 20 μg/mL trypsin in the absence or presence of 1% octylglucoside. The reaction was stopped with the addition of protease inhibitors. After incubation with DNaseI for 5 min, the samples were solubilized with 2 x sample buffer, and electrophoresed on 10% SDS-PAGE.

Cell-based Palmitoylation Assay - COS-1 cells expressing Shh and indicated Hhat constructs were starved for 1 h in DMEM medium containing 2% dialyzed fetal calf serum, followed by incubation with 13 μCi/ml ^{125}I-Iodopalmitate for 4 h at 37°C. Cells were washed twice with 2 ml of ice-cold STE buffer and lysed in radioimmune precipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA). Lysates were clarified by ultracentrifugation at 100,000 × *g* for 15 min in a Beckman T100.2 rotor. Immunoprecipitations were performed by incubating clarified lysates with 7 μl of anti-Shh and 50 μl of protein A/G+ agarose beads (Santa Cruz Biotechnology) for 16 h at

4°C. The beads were washed twice with 500 µl of RIPA buffer, and bead pellets were resuspended in 40 µl of 2 × sample buffer containing 40 mM dithiothreitol (DTT). Immunoprecipitated samples were electrophoresed on a 12.5% SDS-PAGE, dried, and exposed by phosphorimaging for 4 days. Screens were analyzed on a Typhoon™ FLA-7000 bioimaging analyzer (GE Healthcare, Little Chalfont, United Kingdom). Labeling experiments were performed in duplicate and repeated three times.

Protein Stability Assay – COS-1 cells were transfected with the indicated Hhat constructs and, 48 h post transfection, placed in DMEM media supplemented with 10% FBS, 100 µg/ml cycloheximide, and 40 µg/ml chloramphenicol. After incubation for 0 h or 24 h, cells were washed twice with 2 ml of STE and scraped in 500 µl of RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using DC™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) and equal amounts of samples were electrophoresed on SDS-PAGE gels, transferred onto PVDF membranes and probed with anti-HA, anti-Flag, or anti-Myc antibodies. Expression levels were quantified from Western blots using Quantity One (Bio-Rad Laboratories, Inc.) with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Inc.). Experiments were performed in duplicate and repeated three times.

Results

Predicted Membrane Topology of Hhat – We first performed *in silico* analysis of the membrane topology of human Hhat (UniProt # Q5VTY9-1) using 10 different prediction programs. Between 10-13 TMD helices were predicted by these programs, and no signal peptide was evident (Fig. 3.1A). Most TMDs, especially helices 5-13, were predicted with remarkable consistency, with the boundaries of potential TMDs marked within a few residues across 7-10 of the algorithms. There was, however, variability among the different prediction programs in the N-terminal region of Hhat. Specifically, the presence of potential TMDs between residues 87-132 was inconsistent. MemBrain [536] predicted one possible re-entrant helix (residues 96-105) and a closely spaced subsequent TMD. MEMSAT3 [497] predicted the presence of two TMDs, and most other programs predicted one TMD [498, 537-541]. Among the programs that predicted one TMD in this region, there was considerable variation in the boundaries of the TMD helix. Analysis with ZPRED [542], which predicts the distance of a given residue to the center of the membrane, indicated that residues 95-119 were likely located within the membrane bilayer.

Identification of the Membrane Topology of Hhat via Differential Membrane Permeabilization – To experimentally determine the topology of Hhat, Flag and HA epitope tags were introduced within hydrophilic regions between predicted TMDs as well as at either N- or C-terminus (Fig. 3.1B). Each internal epitope insertion construct also contained a C-terminal tag, which allowed us to verify that the epitope insertion does not significantly alter protein topology and 'flip' the C-terminus across the membrane. Flag

insert constructs were designed with an HA C-terminal tag, and HA insert constructs were designed with either a Flag or Myc C-terminal tag. The orientation of each epitope tag with respect to the ER membrane was analyzed via differential membrane permeabilization coupled with immunofluorescence microscopy. COS-7 cells expressing individual Hhat constructs were treated with either digitonin, to selectively permeabilize the plasma membrane, or Triton X-100, to permeabilize all cellular membranes. Cells were incubated with antibodies directed against terminal or internal epitope tags. Co-staining for endogenous protein disulfide isomerase (PDI), an ER protein localized to the ER lumen, provided an internal control to verify either complete or selective membrane permeabilization.

Multiple cytosolic loops were identified using the Flag and HA-tagged constructs. A Flag epitope placed at the N-terminus of Hhat (Flag-HhatHA) exhibited a cytosolic localization, as permeabilization with digitonin was sufficient to detect the epitope tag (Fig. 3.2). Flag epitopes inserted into 6 predicted loops - 92Flag, 124Flag, 233Flag, 320Flag, 402Flag, and 430Flag – also displayed cytosolic orientations (Fig. 3.2, Table 3.1). The cytosolic orientation of both 92Flag and 124Flag epitopes suggests that the hydrophobic segment predicted by most algorithms between residues 87-132 does not cross the membrane. An additional cytosolic loop was identified with the 194HA construct (Fig. 3.3A). Moreover, two HA insert constructs, 230HA and 351HA, contain epitope inserts within the same predicted loops as the 233Flag and 320Flag constructs, respectively. Both 230HA and 351HA displayed cytosolic orientations, consistent with the results from the respective Flag insert constructs (Fig. 3.3A, Table 3.1). The cytosolic

A

Prediction Program	TMD 1	TMD 2	TMD 3	TMD 4	TMD 5	TMD 6	TMD 7	TMD 8	TMD 9	TMD 10	TMD 11	TMD 12	TMD 13
MemBrain (20)	6-22	68-84	96-105	107-129	132-148	163-183	203-217	244-271	282-310	364-380	384-399	428-448	463-481
MEMSAT3 (21)	7-26		87-110	113-132	135-154	165-184		244-268	284-304	365-384	387-406	429-449	468-487
MEMSAT-SVM (22)	8-24	68-94	98-128		132-150	166-183	205-221	244-269	285-313	363-379	383-402	427-457	462-485
SCAMPI-seq (23)	2-22	69-89	99-119		130-150			239-259	293-313	360-380	382-402	429-449	460-480
SCAMPI-msa (23)	7-27		95-115		129-149	165-185	202-222	244-264	293-313	360-380	382-402	427-447	460-480
PRODIV (24)	7-27	68-88	98-118		127-147	168-188	200-220	241-261	293-313	345-365	386-406	427-447	468-488
PRO (24)	7-27	70-90		102-122	127-147	168-188	201-221	242-262	283-303	361-381	386-406	427-447	464-484
OCTOPUS (25)	7-27		95-115		129-149	165-185	202-222	244-264	293-313	360-380	382-402	427-447	460-480
TOPCONS (26)	7-27	69-89	98-118		129-149	168-188	201-221	242-262	293-313	360-380	382-402	427-447	460-480
TMFinder (27)	6-24	70-85			112-148	169-178		241-272	284-313	365-377	386-395	434-445	457-488

	TMD 1	TMD 2	RL 1	TMD 3	TMD 4	RL 2	TMD 5	TMD 6	TMD 7	TMD 8	TMD 9	TMD 10
Final Topology Model	6-22	68-84	95-119	132-148	163-183	203-217	244-271	282-310	364-380	384-399	428-448	463-481

B

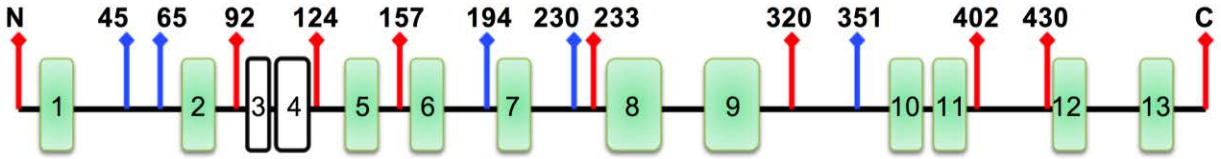

Figure 3.1 Predicted transmembrane domains for human Hhat. A, Transmembrane domains (TMDs) predicted for human Hhat by the indicated programs (top panel). TMDs predicted with high consistency by most programs are highlighted in green. Final topology model of Hhat (bottom panel) RL, re-entrant loop. B, schematic representation of Flag epitope insert (red arrows) and HA epitope insert (blue arrows) constructs used to map the topology of Hhat.

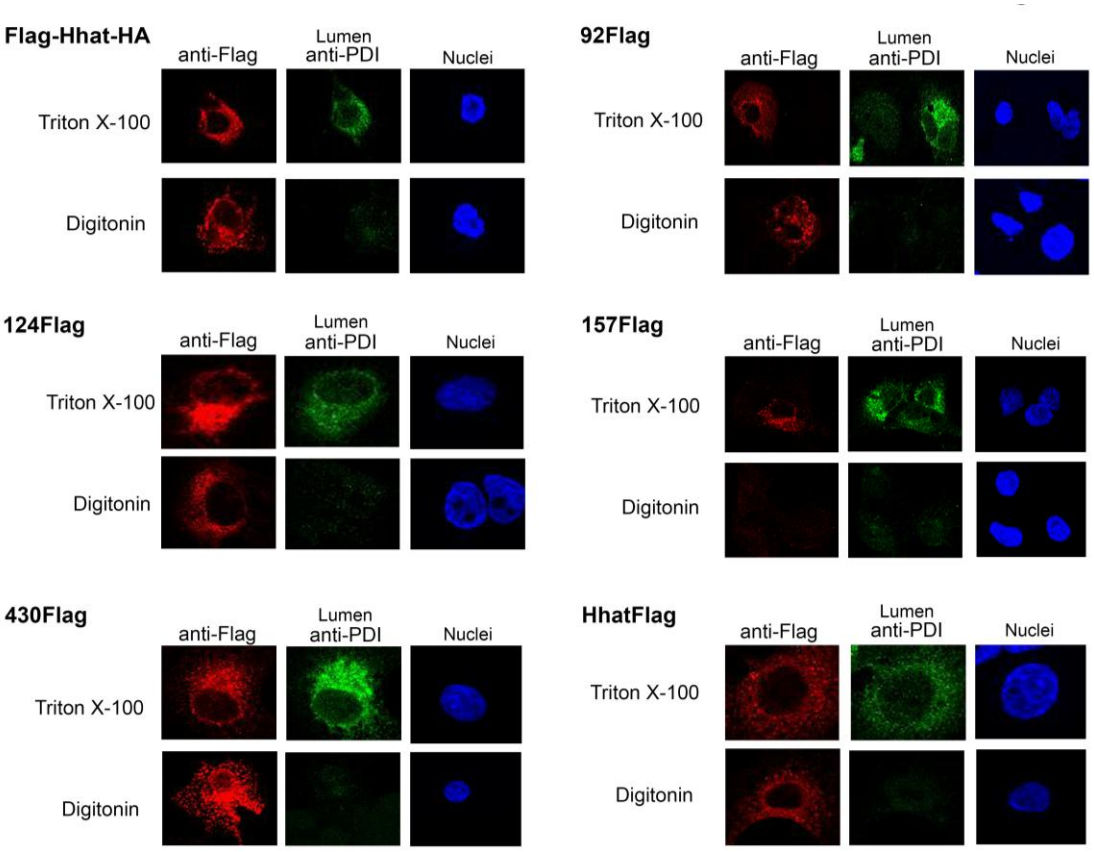

Figure 3.2 Hhat topology mapping using Flag insert constructs. COS-7 cells were transfected with Hhat cDNA containing the indicated internal Flag epitope tags. Digitonin was used to selectively permeabilize the plasma membrane and Triton X-100 was used to permeabilize all cellular membranes. Cells were stained with anti-Flag antibody to visualize the cytosolic or lumenal orientation of the inserted Flag tag. Staining of endogenous PDI served as an internal control for a lumenal epitope. Images for each construct were collected using the same conditions on the same day ensuring fair side-by-side comparison.

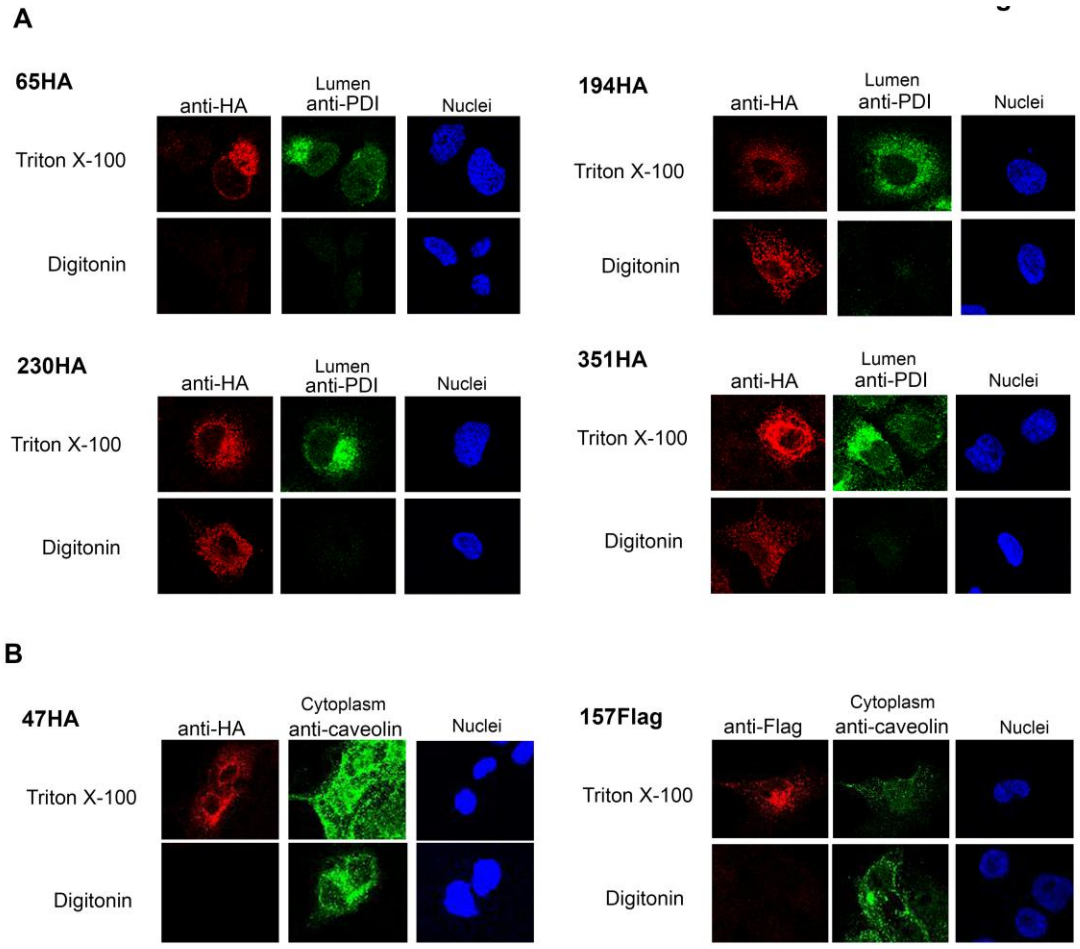

Figure 3.3 Hhat topology mapping using HA insert constructs. A, COS-7 cells were transfected with Hhat cDNA containing the indicated internal HA epitope tags. Cells were treated and visualized as in Figure 2. B, COS-7 cells were transfected with Hhat cDNA with the indicated internal Flag or HA epitope tags. Cells were treated as in Figure 2 with one exception: staining of endogenous caveolin served as an internal control for a cytosolic epitope. Images for each construct were collected using the same conditions on the same day ensuring fair side-by-side comparison

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TABLE 3.1
Summary of Hhat Membrane Topology Experiments

Epitope Tag	Location: IF Assay[a]	Location: Protease Assay[b]
N-term Flag	Cytosol	ND[c]
47HA	Lumen	ND
65HA	Lumen	ND
92Flag	Cytosol	Cytosol
124Flag	Cytosol	Cytosol
157Flag	Lumen	Lumen
194HA	Cytosol	ND
230HA	Cytosol	ND
233Flag	Cytosol	ND
320Flag	Cytosol	ND
351HA	Cytosol	ND
402Flag	Cytosol	ND
430Flag	Cytosol	Cytosol
C-term Flag	Cytosol	Cytosol
C-term Myc	ND	Cytosol

[a] IF (immunofluorescence) assay as described for Figures 2 and 3
[b] Protease protection assay as described for Figure 4
[c] ND, not determined

orientation of epitope tags on either side of the strongly predicted hydrophobic segment from residues 203-217 suggests that this segment does not span the membrane. In addition, these data localize the highly conserved Asp339 residue to the cytosol. Finally, a Flag tag at the C-terminus of Hhat exhibited a cytosolic orientation (Fig. 3.2).

Lumenal loops were identified when permeabilization with Triton X-100, but not with digitonin, was required to detect the epitope tag insertion. These included 157Flag as well as 47HA and 65HA, which have HA tags within the same predicted loop (Figs. 3.2, 3.3). Co-staining with an antibody directed against an endogenous caveolin epitope localized in the cytoplasm was also performed. In all cases, caveolin fluorescence was detected with digitonin as well as Triton X-100, confirming that selective permeabilization of the plasma membrane was achieved (Fig. 3.3B). Finally, for each construct with an internal epitope insertion, the C-terminal tag was stained in parallel and confirmed to be localized in the cytosol, suggesting that the overall topology of Hhat was not affected by the internal epitope tag insertion. Taken together, these data suggest that both N and C termini of Hhat are located in the cytosol and establish the presence of six cytosolic and two lumenal loops within Hhat.

Identification of the Membrane Topology of Hhat via Protease Protection Assay – We next used an *in vitro* protease protection assay as an additional method to analyze the orientation of each epitope tag insertion with respect to the membrane. Lumenal epitopes should be protected from protease digestion in the absence, but not the presence of, detergent. Membranes from 293FT cells expressing individual constructs were incubated

with trypsin in the presence or absence of octylglucoside. We chose this detergent as it allows for the purification of an active, functional Hhat [19]. PDI served as an internal control for the integrity of membrane preparations and confirmation of membrane permeabilization upon treatment with octylglucoside. Flag epitope tags within the 92Flag, 124Flag, and 430Flag constructs were cytosolic, as incubation with trypsin alone was sufficient to digest the epitope tag (Fig. 3.4A,B,D). In contrast, the Flag epitope in the 157Flag construct was lumenal, as the epitope was only accessible to trypsin-mediated digestion upon membrane permeabilization with octylglucoside (Fig. 3.4C). Constructs with a C-terminal Flag or Myc tag displayed a cytosolic orientation (Fig. 3.4E,F). These data are consistent with the findings from the immunofluorescence-based assay. Use of the remaining Flag insert constructs in the protease protection assay generated inconsistent results, which may reflect changes in epitope accessibility or topology resulting from the membrane preparation. Furthermore, HA insert constructs could not be used in this assay as we were unable to detect the HA tags via immunoblotting, despite being able to detect the tags by immunofluorescence. A similar issue with epitope recognition was observed with a subset of internal epitope tags within GOAT [282].

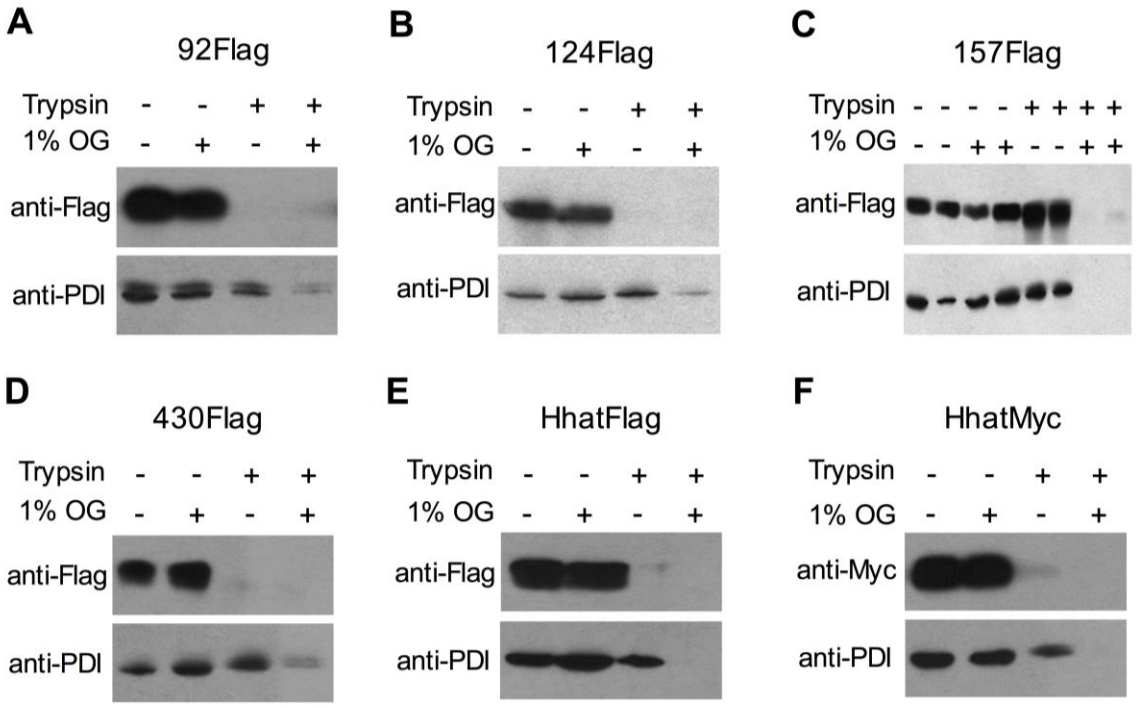

Figure 3.4 Protease protection assay to determine the topology of Hhat. A-F, Membranes were prepared from 293FT cells transfected with (A) 92Flag, (B) 124Flag, (C) 157Flag, (D) 430Flag, (E) HhatFlag, or (F) HhatMyc constructs. Membranes were incubated with trypsin in the absence or presence of 1% octylglucoside (OG). Samples were electrophoresed on 10% SDS-PAGE and probed with the indicated antibodies.

Activity and Stability of Hhat Proteins with Internal Epitope Tags – To determine whether epitope tag insertions resulted in gross changes in protein structure or function, we compared the activity and stability of individual constructs to that of Hhat with the corresponding C-terminal tag. We monitored the catalytic activity of each construct using a cell based palmitoylation assay. COS-1 cells were co-transfected with cDNAs encoding Shh and either empty vector, C-terminally tagged Hhat, or individual epitope insertion Hhat constructs. Cells were then labeled with ^{125}I-Iodopalmitate, a radioiodinated palmitate analog. Shh was immunoprecipitated from cell lysates and the amount of radiolabeled palmitate incorporation into Shh was determined by phosphorimaging analysis after SDS-PAGE.

Hhat constructs with Flag epitopes at the N-terminus or within the N-terminal half of Hhat, with the exception of 124Flag, retained activity comparable to that of Hhat. In contrast, Flag insertions within the C-terminal portion of Hhat exhibited significantly decreased activity (Fig. 3.5A). Four out of five HA insert constructs showed activity comparable to that of HhatFlag or HhatMyc (Fig. 3.5B). Consistent with the behavior of the Flag insert constructs, the HA insert closest to the C-terminus – 351HA – was also compromised in its catalytic activity (Fig. 3.5B). Importantly, the subcellular localization of each construct was comparable to that of wildtype Hhat (Figs. 3.2, 3.3). The lack of large puncta or aggregates suggests that the reduced catalytic activity of a subset of constructs was not due to gross protein misfolding.

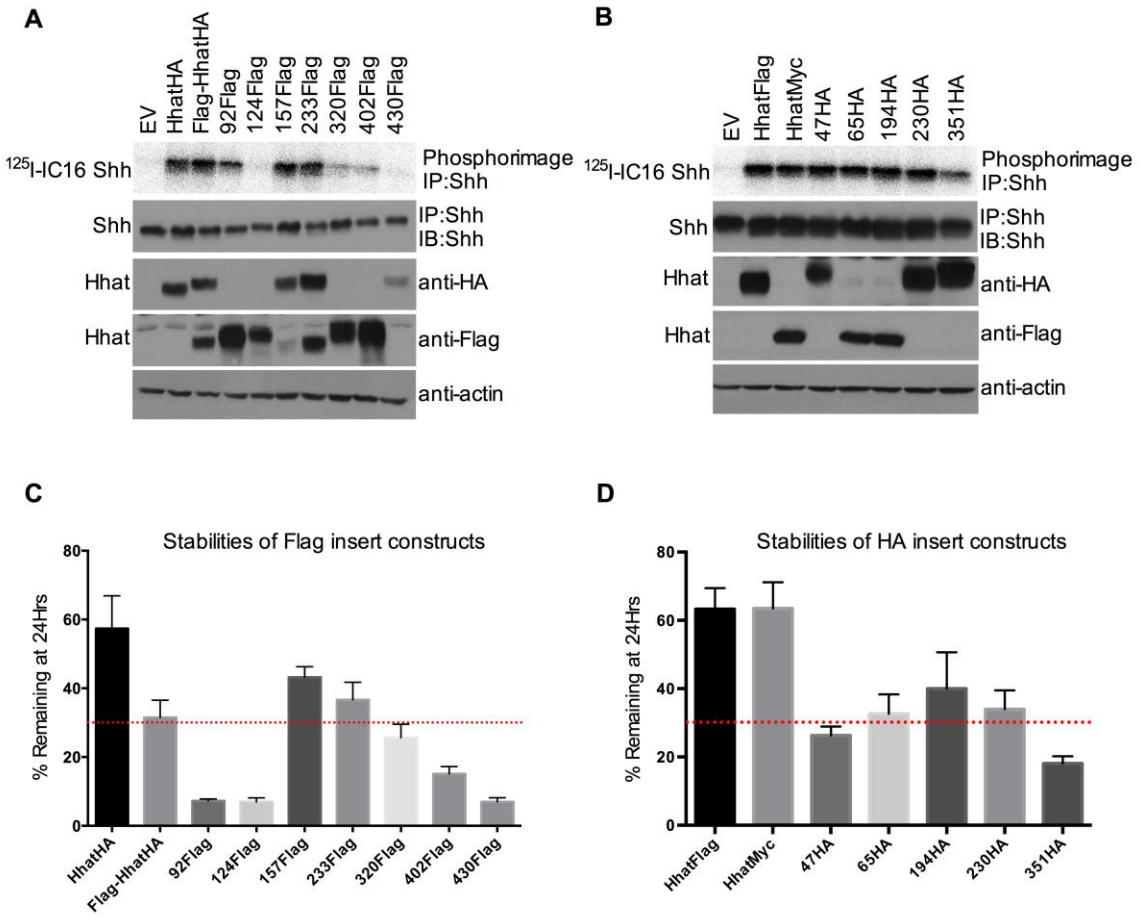

Figure 3.5 Activity and stability of constructs used to map the topology of Hhat. A-B, COS-1 cells co-expressing Shh and Hhat with the indicated Flag insert (A) or HA insert (B) were labeled with ^{125}I-Iodopalmitate (^{125}I-IC16) for 4 h. Cell lysates were immunoprecipitated (IP) with anti-Shh antibody and analyzed by SDS-PAGE and phosphorimaging (top panels) or Western blotting with anti-HA, anti-Flag, anti-Myc or anti-actin antibodies. The experiment was performed three times in duplicate; a representative image is shown. C-D, COS-1 cells expressing the indicated Flag insert (C) or HA insert (D) Hhat constructs were treated with 100μg/mL of cycloheximide and 40μg/mL chloramphenicol for 0 h or 24 h. Cells were lysed and analyzed by Western blotting with an antibody directed against the C-terminal tag (anti-HA, anti-Flag, or anti-Myc) after SDS-PAGE. The amount of Hhat signal at each time point was determined by densitometry. Each point indicates the mean ± S.D. (n = 3).

We noted variability in the detection of each Flag insert construct by immunoblotting (Fig. 3.5A). Two constructs – Flag-HhatHA and 233Flag – were detected by both HA and Flag antibodies, while the remaining constructs could only be detected by either the Flag or HA antibody. With the exception of 430Flag, constructs that could be detected by the HA antibody exhibited comparable expression to that of HhatHA. Expression of the HA insert constructs was monitored by their C-terminal tags as internal HA epitopes were undetectable by immunoblotting. HA insert constructs showed comparable expression to that of HhatFlag or HhatMyc (Fig. 3.5B). Taken together, these data suggest that epitope insertions within the N-terminal portion of Hhat are well tolerated whereas insertions within the C-terminal MBOAT homology domain lead to reduced activity.

We next asked whether decreased stability could account for the reduced catalytic activity exhibited by some constructs. COS-1 cells expressing individual epitope insertion constructs were treated with cycloheximide and chloramphenicol to block new protein synthesis, and levels of protein remaining at 24 h were quantified. Hhat with a C-terminal HA, Flag, or Myc tag was remarkably stable with about 57-63% of the initial protein remaining after 24 h (Fig. 3.5C,D). Three constructs carrying Flag epitope insertions within the N-terminal half of Hhat (Flag-HhatHA, 157Flag, and 233Flag) were about half as stable as HhatHA. Importantly, the catalytic activity of these constructs was not compromised (Fig. 3.5A). The 92Flag and 124Flag constructs exhibited reduced stability with less than 10% of initial levels of protein remaining after 24 h (Fig. 3.5C). Despite low stability, the catalytic activity of 92Flag was not affected (Fig. 3.5A). In contrast, the decreased stability of 124Flag may account for its reduced activity (Fig.

3.5A,C). Furthermore, Flag insertions within the C-terminal portion of Hhat also led to reduced stability, suggesting reduced stability may account for the reduced activity of these constructs (Fig. 3.5A,C). HA epitope insertions led to a twofold decrease in stability compared to HhatFlag or HhatMyc (Fig. 3.5D), however, most of these proteins remained catalytically active (Fig. 3.5B). 351HA, which exhibited impaired catalytic activity, also displayed a modest reduction in stability compared to the other HA insert constructs. Taken together, these data suggest that while epitope insertions result in reduced stability, they do not necessarily lead to a corresponding decrease in activity.

Discussion

Here we present an experimentally derived model for the membrane topology of Hhat, using selective permeabilization coupled with immunofluorescence as well as a protease protection assay. We determined that both N and C termini of Hhat are localized in the cytosol, and identified ten TMDs and two re-entrant loops. These features are incorporated into the topology model depicted in Fig.3.6. Most of the Hhat TMDs, especially helices 3-10 (Fig. 3.6), were predicted with high consistency by multiple algorithms. This region encompasses the MBOAT homology region and contains the presumed active site. The invariant His379 residue is located between two adjacent TMD helices, at the boundary of the membrane and the lumen of the ER. The orientation of His379 within the lumenal side of the membrane is consistent with its role in Hhat catalysis [281]. This organization is also observed in the recently published topology model for GOAT [282] and may provide a favorable hydrophobic environment for the

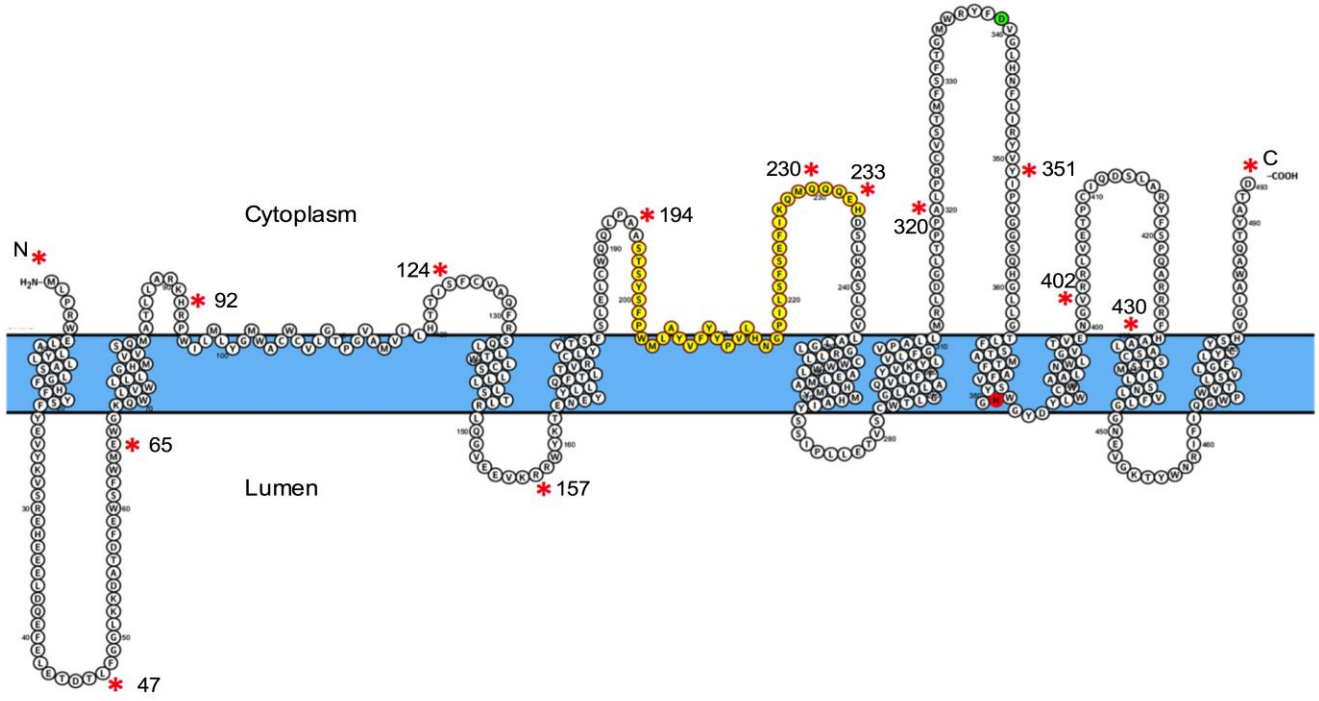

Figure 3.6 Model for the membrane topology of Hhat. Graphical representation of the topology of human Hhat generated with Protter Server [543]. The boundaries of TMDs were designated using output from MemBrain [536]. The boundaries of the first re-entrant loop (residues 95-119) are determined as discussed in the text. The area of high conservation (residues 196-234) among MBOAT enzymes with protein substrates is highlighted in yellow. The invariant His379 residue is shown in red and the highly conserved Asp339 is shown in green. Positions of Flag epitope and HA epitope insertions (asterisks) are indicated along with the corresponding residue numbers.

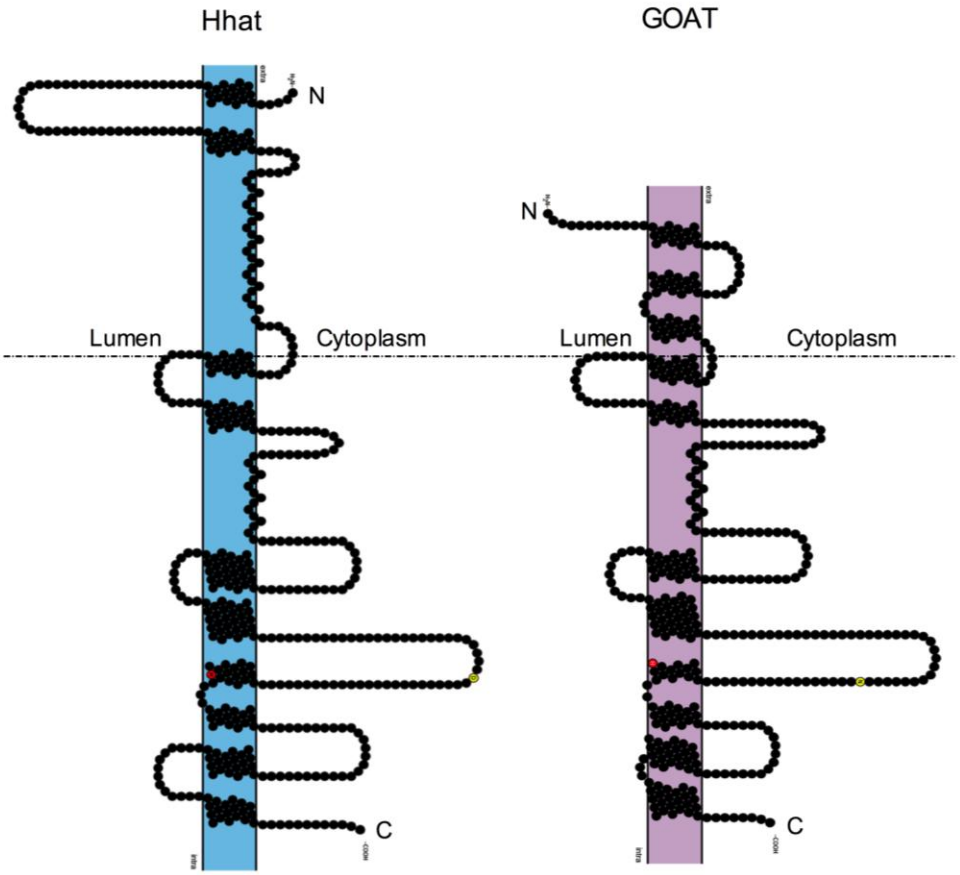

Figure 3.7 Hhat and GOAT have similar membrane topologies. Graphical representation of the topology of human Hhat (left) and human GOAT as previously described [282] (right) generated with Protter Server. The conserved His and Asp residues are shown in red and yellow, respectively. The topological organization of the C-terminal regions of Hhat and GOAT are highly conserved (below dotted line).

catalytic reaction which uses fatty acyl-CoA as a substrate. However, the highly conserved Asp339 residue is located in the cytoplasm. The tightly packed helices around the invariant His residue and the segregation of the conserved His and Asp residues on opposite sides of the membrane was also observed in the topology model for GOAT.

A striking aspect of Hhat topology is the remarkable similarity to the topology of GOAT (Fig. 3.7). The overall disposition and location of the TMDs and loops is nearly identical over two thirds of the Hhat and GOAT proteins, despite <20% primary sequence identity. In addition, we note that a large fraction of the protein is located within the cytoplasm (172 cytoplasmic residues vs. 87 lumenal residues). Similarly, a to-scale model of GOAT topology reveals that it has long loops in the cytoplasm (161 residues in total) and mostly smaller loops (56 residues in total) in the lumen. The large cytoplasmic loops of these MBOAT enzymes may be required for protein-protein interactions, regulatory modifications or substrate binding.

Due to the ambiguity in the predictions for TMDs between residues 87-132, we designed two constructs, 92Flag and 124Flag, in this region (Fig. 3.1). Both Flag epitopes were determined to be cytosolic by two independent assays suggesting that this hydrophobic stretch of residues does not cross the membrane (Figs. 3.2, 3.4). Although the 92Flag insertion resulted in an unstable protein, the catalytic activity was not affected (Fig. 3.5). In contrast, the 124Flag insertion resulted in a significant decrease in both stability and activity (Fig. 3.5). Although it remains possible that this Flag tag interrupts a TMD as predicted by MemBrain and MEMSAT-SVM programs, all other algorithms place residue 124 outside potential TMDs (Fig. 3.1). Furthermore, the subcellular localization of the 124Flag protein was comparable to that of wildtype Hhat and the C-

terminus of the construct was localized in the cytosol, suggesting that the membrane insertion and overall topology of this construct is not affected. Based on our data, it is likely that this region forms either a re-entrant loop or folds into a small domain with a hydrophobic interior within the cytosol. We propose that Hhat contains a re-entrant loop between residues 95-119, which is consistent with the ZPRED analysis indicating that these residues are located within the membrane. Three-dimensional structural determination will be required to provide definitive confirmation for this interesting structural feature within Hhat.

We also identified the presence of a second re-entrant loop between residues 203-217 of Hhat. Epitope insertions on either side of this hydrophobic segment (194HA, 230HA, and 233Flag) were localized in the cytosol suggesting the hydrophobic segment does not cross the membrane (Fig. 3.3, Table 3.1). Importantly, these insertions do not alter the catalytic activity of Hhat (Fig. 3.5) suggesting that the tertiary structure of the protein is unaltered. It is possible that these residues form an interfacial helix, as the amino acid sequence of this segment (**W**MLA**Y**VF**YY**PVLHNG) contains multiple Tyr and Trp residues, known to be enriched in interfacial helices [487]. Interestingly, global sequence alignment of Hhat, Porcn, and GOAT identified a second region of high conservation, located between residues 196 and 234 in Hhat [281]. This region encompasses the re-entrant loops within Hhat and GOAT, and sequence similarity suggests that Porcn may also contain a re-entrant loop in this region. In addition, mutation of specific residues (Y207A and G217A) within this region of Hhat reduces catalytic activity [281]. Further structure-function studies are needed to determine

whether a re-entrant loop in MBOAT enzymes with protein substrates plays a role in substrate recognition, protein stability, and/or catalysis.

Acknowledgements

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Footnotes

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Chapter Four

Conclusions

In this thesis, we show a functional significance for Hhat activity in specific subtypes of breast cancer cells. Depletion of Hhat with lentiviral shRNA decreased both anchorage-dependent and anchorage-independent proliferation of ER positive, but not triple negative, breast cancer cells. Treatment with RU-SKI 43, a small molecule inhibitor of Hhat, also reduced proliferation of these cells. Furthermore, depletion or inhibition of Hhat reduced the proliferation of HER2 amplified as well as tamoxifen resistant cells. Surprisingly, we found that Hhat regulated the proliferation of both Shh responsive and non-responsive ER positive cells, suggesting a Shh independent function for Hhat. Together, these data suggest that Hhat plays a critical role in ER positive, HER2 amplified, and hormone resistant breast cancer proliferation and highlights the potential promise of Hhat inhibitors for therapeutic benefit.

Parallel to these efforts, we also conducted a topological analysis of Hhat. Bioinformatics analysis of transmembrane domains within human Hhat using ten different algorithms resulted in highly consistent predictions in the C-terminal, but not in the N-terminal, region of Hhat. Combining this analysis with empirical methods, we demonstrated that Hhat contains ten transmembrane domains and two re-entrant loops. The invariant His and highly conserved Asp residues within the MBOAT homology domain are segregated on opposite sides of the endoplasmic reticulum membrane. The localization of His379 on the lumenal membrane surface is consistent with a role for this invariant residue in catalysis. Knowledge of the topological organization of Hhat could serve as an important tool for further design of selective Hhat inhibitors.

Future Directions

Investigate additional functions of Hhat in breast cancer

Hhat is required for the proliferation of ER positive, HER2 amplified, or tamoxifen resistant cells. Further experiments are needed to determine whether Hhat is required for the growth of these types of tumors *in vivo*. Moreover, Shh signaling is known to mediate not only proliferation but also migration/invasion and stem cell maintenance [13, 364, 447], and the specific contribution of Hhat to these processes has not been elucidated. One report indicates a role for Hhat in stem cell maintenance of lung squamous cell carcinomas [25]. In this context, Hhat expression is upregulated by SOX2 mediated transcription specifically in the cancer stem cells. Furthermore, reducing Hhat expression or inhibiting its activity results in a significant decrease in stem cell numbers

and a consequent reduction of tumor growth *in vivo*. Hhat regulates stem cell maintenance through its effects on Shh signaling, as Gli1 activation is required for this process [25]. Given our findings that Hhat can have Shh independent functions, it will be interesting to see whether Hhat can modulate migration/invasion or stem cell maintenance in a Shh independent manner.

a) Tumor growth *in vivo*: MCF7 or BT474 cells are frequently used in xenograft models of breast cancer and represent ER positive and ER positive/HER2 amplified cells, respectively [544, 545]. Cells stably expressing control scrambled or Hhat specific shRNA can be suspended with reconstituted basement membrane (Matrigel) in 1:1 volume and injected subcutaneously into 6 to 8-week old female BALB/c athymic mice pre-implanted with β-estradiol pellets. Tumor sections can be fixed and stained with hematoxylin and eosin as well as with antibodies directed against Shh and Gli1 to determine whether tumor or stromal cells display differences in Shh signaling. Alternatively, cells can be orthotopically injected into the mammary fat pad to mimic the appropriate niche for tumor growth.

To test the efficacy of an Hhat inhibitor, mice can be treated with a vehicle control or an Hhat inhibitor after tumors are allowed to form. Pharmacodynamic analysis of RU-SKI 43 in mice indicates that this compound has a short *in vivo* half-life (~17 min). Therefore, the development of second generation compounds, which retain inhibitory activity and display increased *in vivo* stability, are required. Such an Hhat inhibitor can also be tested in combination with mTOR/PI3K inhibitors, ER modulators,

or anti-HER2 therapies to determine whether Hhat inhibitors enhance the cytotoxic effects of current treatment options *in vivo*.

b) Cell migration: Breast cancer cells can be plated in serum free media, with or without Hhat inhibitors, in the upper compartment of transwell chambers. The lower chamber would contain media with serum and cells migrating to the lower side of the membrane can be fixed, stained with Giemsa and counted. These experiments can also be conducted using cells stably expressing control scrambled or Hhat specific shRNA.

c) Cell invasion: To assay invasiveness in the absence or presence of Hhat inhibition, breast cancer cells in serum free media can be plated in the top chamber of transwells coated with Matrigel. Media with serum or conditioned media from fibroblasts, which is enriched in growth factors, would be added to the lower chamber. The percentage of invading cells can be calculated in the presence and absence of Hhat inhibitors or with cells stably expressing control scrambled or Hhat specific shRNAs.

d) Metastasis: Since T47D and MCF7 cells are poorly metastatic, BT474 or MDA-MB-453 cells should be used to monitor the role of Hhat in tumor invasiveness *in vivo* [546]. Breast cancer cells stably expressing control scrambled or Hhat specific shRNAs can be introduced into the blood stream of 6 to 8-week old female BALB/c athymic mice via tail vein inoculation. Five weeks post injection, lungs can be dissected, fixed, stained with

hematoxylin and eosin, and tumor foci will be quantified. As a more stringent test of metastasis, cells can be orthotopically transplanted into the mammary fat pad of $Rag2^{-/-}$;$Il2rg^{-/-}$ mice [546]. A complete necropsy examination can be performed to confirm the presence of metastatic lesions specifically in the lungs, bones, and brain.

e) Stem cell maintenance: Cancer stem cells can be isolated from cell lines or primary tumors via fluorescence-activated cell sorting based on previously established surface markers ($CD45^{-}Ter119^{-}CD39^{-}Sca\text{-}1^{low}CD24^{med}CD49f^{high}$) [443, 444]. The stemlike phenotype of these cells can be verified by monitoring tumor sphere formation, serial passage capacity and *in vivo* tumorigenicity. Hhat expression can be compared between the cancer stem cells and the bulk of the tumor. However, even low levels of Hhat expression are enough to mediate catalysis. Therefore, to analyze the functional significance of Hhat, the effect of shRNA mediated knockdown or pharmacological inhibition of Hhat should be monitored. The effect of knockdown or inhibition of Hhat on cell cycle, tumor sphere formation, serial passage capacity, and *in vivo* tumorigenicity should be tested. In addition, monitoring expression of cell surface markers with flow cytometry can determine whether the cancer stem cells undergo differentiation upon Hhat knockdown or inhibition.

Identification of novel substrates of Hhat

Our data indicates that Hhat has functions beyond mediating Hh signaling. We demonstrated that MCF7 and CAMA-1 breast cancer cells require Hhat, but not Shh, for proliferation, suggesting the presence of additional substrates for Hhat (Figs. 2.2, 2.4, 2.5, 2.7). Interestingly, Rasp, the Hhat ortholog in *Drosophila*, palmitoylates not only Hh but also Spitz, an EGFR like ligand [259]. Therefore, it is possible that mammalian Hhat also has substrates in addition to Hh ligands. Identification of novel substrates for Hhat is critical to understanding Hhat function in breast cancer.

Comparing the palmitoylated proteomes from cells treated with either vehicle or an Hhat inhibitor will identify potential substrates [547, 548]. First, MCF7 and CAMA-1 cells can be differentially labeled using stable isotope labeling by amino acids in cell culture (SILAC). Cells can be cultured in light ($^{12}C_6$-Lys) or heavy ($^{13}C_6$-Lys) media to ensure incorporation of the heavy amino acids. Cells grown in light media would be treated with DMSO and alkyne-palmitate and cells grown in heavy media would be treated with RU-SKI 43 and alkyne-palmitate in the presence of 2% dialyzed serum for 4 h. Cell lysates would then be mixed in a 1:1 ratio. Samples can be enriched for palmitoylated proteins by streptavidin-agarose bead pull down following a copper-catalyzed azide alkyne cycloaddition. Mass spectrometry will allow for the quantification of the relative abundance of each palmitoylated protein in the two cell populations, as reflected by the signal intensities from the light and heavy samples. Shh from MCF7 cells can serve as a positive control, and potential substrates could be further analyzed using established cell based assays for Hhat activity.

Identification of the binding site of RU-SKI 43

Hhat is required for the proliferation of pancreatic, lung, and breast cancer cells, and therefore it is an attractive drug target for the treatment of these tumors [24-27, Chapter 3]. Our laboratory has identified a specific, small molecule inhibitor of Hhat, RU-SKI 43 [459]. A critical step in understanding how RU-SKI 43 inhibits Hhat is to identify the binding site of the molecule. Furthermore, this information, combined with the topological organization of Hhat, will also establish portions of the protein which are required for enzymatic activity regulation.

To determine the binding site of inhibitors to Hhat, one can use inhibitors crosslinked to benzophenone (BP). BP is a photo affinity probe that is activated by ultraviolet light and results in the formation of a carbon based covalent bond with the target protein. Importantly, ultraviolet exposure does not damage the protein itself. Inhibition of Hhat activity by crosslinked and the corresponding non-crosslinked inhibitors can be quantified *in vitro*. BP has been used to identify binding sites of other inhibitors, i.e. ones that target hepatitis C virus RNA polymerase and HIV-1 integrase, without loss of inhibitory activity [549-551]. Therefore, BP crosslinking is unlikely to affect the function of Hhat inhibitors.

Crosslinked and non-crosslinked inhibitors can be incubated *in vitro* with either membranes from cells expressing Hhat or purified Hhat protein. Saturating levels of inhibitor should be used to ensure efficient binding to Hhat. After incubation with the inhibitors, samples can be exposed to ultraviolet radiation to alkylate the BP crosslinked inhibitors to Hhat. Samples would be separated by SDS-PAGE, the band corresponding to Hhat can be subjected to tryptic digest and analyzed by MALDI-MS. Non-activated

samples can serve as controls. Labeled peptides that show a difference in ultraviolet absorbance compared with the control, can be isolated and the modified residue(s) identified [550].

Once a putative binding site residue has been identified, that residue can be altered using site directed mutagenesis and the activity of the Hhat point mutant determined using an *in vitro* Shh palmitoylation assay. If Hhat activity is retained, then we predict that Hhat inhibitors should no longer be able to reduce activity of the mutant protein. Alternatively, if the mutant Hhat is inactive, it would imply that the binding site for Hhat inhibitors lies within a critical active site residue of Hhat.

The function of large cytoplasmic loops within Hhat

Comparison of Hhat and GOAT membrane topology reveals that a large fraction of both enzymes is located within the cytoplasm, despite the fact that catalysis is thought to occur in the lumen of the endoplasmic reticulum (Fig. 3.7). These large cytoplasmic loops may be required for protein-protein interactions or regulatory modifications. To identify potential interacting proteins or substrates, a Glutathione-S-transferase (GST) fusion protein pull-down approach can be used. Sequences of each cytoplasmic loop fused to GST can be expressed in bacteria and purified using glutathione-agarose beads. After incubation with the cytoplasmic fraction of cell lysates, protein complexes can be eluted from the beads and analyzed by SDS-PAGE and silver staining. Specific bands can be analyzed by mass spectrometry to identify potential protein interactions [552].

In addition, analysis of purified Hhat with mass spectrometry can be used to identify potential post-translational modifications. These potential modifications can then be verified using mutational analyses and further examined for their potential impact on catalysis or protein-protein interactions. Currently, one study has suggested that Hhat may contain such modifications. Konitsiotis and colleagues [553] recently reported that Hhat is palmitoylated, as simultaneous mutation of four cytosolic Cys residues led to decreased palmitoylation. However, the palmitoylation signal was weak in this study and may represent the autoacylated intermediate. When we labeled COS-1 cells overexpressing Hhat with ^{125}I-Iodopalmitate, no significant incorporation of palmitate into Hhat was detected (Fig. 4.1). We did, however, notice a labeled higher migrating band, suggesting the presence of a possible Hhat multimer.

Does Hhat form multimers?

We have also noted the presence of higher molecular weight migrating bands when detecting Hhat using various antibodies (Fig. 4.2A). To further investigate the presence of potential multimers, we co-expressed HhatFlag and HhatMyc and monitored their interaction by co-immunoprecipitation. We detected interaction between HhatFLag and HhatMyc in lysates from cells expressing both proteins suggesting the presence of oligomers (Fig. 4.2B). Importantly, mixing lysates from cells expressing either one of the proteins did not result in co-immunoprecipitation (Fig. 4.2B, lane 4). Oligomerization of other MBOAT enzymes, such as ACAT and DGAT, has been reported to modulate catalytic activity [503, 554, 555]. Therefore, determining whether multimerization also plays a role in regulating Hhat activity will be interesting.

Hhat multimerization can be verified using crosslinking reagents. Cells expressing Hhat can be treated with increasing concentrations of a membrane permeable crosslinker such as disuccinimidyl suberate. Lysates can be analyzed by SDS-PAGE to determine whether there is a change in the ratio of monomer to dimer/tetramer (45kDa to 90kDa/180kDa). Furthermore, lysates from cells expressing Hhat can be subjected to sucrose gradient sedimentation and fractionation. If separation of higher molecular weight oligomers from monomeric forms is successful, one can compare the activity of the two forms using an *in vitro* Shh palmitoylation assay.

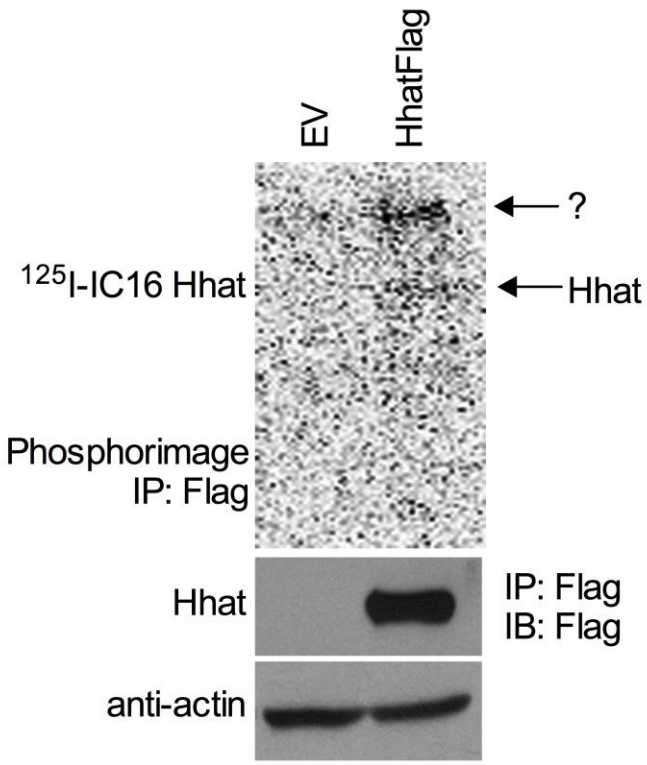

Figure 4.1 Lack of palmitate incorporation into Hhat. COS-1 cells expressing either empty vector (EV) or HhatFlag were labeled with ^{125}I-Iodopalmitate (^{125}I-IC16) for 4 h. Cell lysates were immunoprecipitated (IP) with an anti-Flag antibody and analyzed by SDS-PAGE and phosphorimaging (top panel) or Western blotting with anti-Flag or anti-actin antibodies. The 'Hhat' arrow indicates the position of monomeric Hhat. The '?' arrow indicates the migration position of a possible Hhat multimer. The experiment was performed once in duplicate.

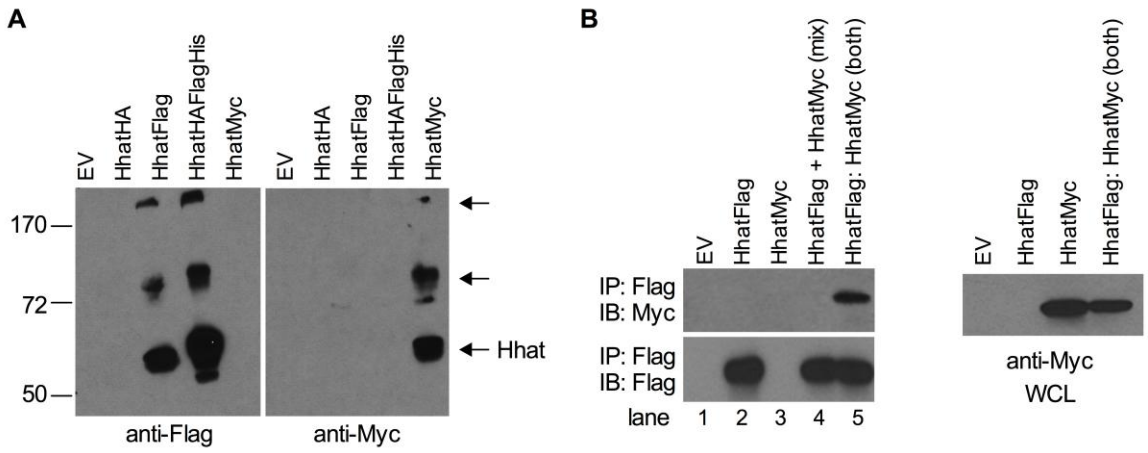

Figure 4.2 Possible multimerization of Hhat. A, Membranes were prepared from 293FT cells transfected with empty vector (EV) or the indicated Hhat constructs. Samples were electrophoresed on SDS-PAGE and probed with the indicated antibodies. The 'Hhat' arrow indicates the position of monomeric Hhat. The top two arrows indicate potential multimers of Hhat. B, *left panel,* COS-1 cells were transfected with empty vector, HhatFlag, HhatMyc, or with both HhatFlag and HhatMyc (lane 5). As a control, lysates from cells transfected separately with HhatFlag or HhatMyc were mixed (lane 4). Lysates were immunoprecipitated (IP) with an anti-Flag antibody and analyzed by Western blotting with anti-Myc or anti-Flag antibodies. *right panel,* whole cell lysates from cells transfected with the indicated constructs and probed with anti-Myc. The experiment was performed twice in duplicate.

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