

The Role of Hedgehog Acyltransferase & Heparan Sulphate Proteoglycans in Human Sonic Hedgehog Signalling

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ABSTRACT

Hedgehog proteins (Hh) are morphogens and major mediators in many developmental processes. Hh signalling is significant for many aspects of embryonic development, whereas dysregulation of this pathway is associated with several types of cancers. Hh proteins require dual lipidation and Heparan Sulfate Proteoglycans (HSPGs) for their proper distribution and signalling activity.

My first aim was to study the role of HSPGs in human (h) Sonic Hedgehog (Shh) signalling and clarify the biological function of hShh/HSPGs complexes in hShh signalling, by investigating the interaction between human hShh and HSPGs. I used DNA mutagenesis and heparin affinity chromatography to determine key residues in hShh involved in heparin binding (K37/38 and K178). The activity of these mutants was tested by detecting induced Alkaline Phosphatase activity in *C3H10T1/2* cells and hShh-inducible gene expression in PANC1 human pancreatic carcinoma cells. I examined the biological function of mutated hShhs (K37/38S, K178S and K37/38/178S) that cannot interact with heparin efficiently and showed that they had reduced signalling activity compared to wild type hShh and a control mutation (K74S). Also, I showed that mutant hShh proteins mediate reduced proliferation and invasion of PANC1 cells following hShh RNAi knockdown (KD), and this correlated with reduced Shh multimeric complex formation.

Structurally, Shh proteins are unusual in being dually lipid-modified to be fully

active. During the post-translational modifications of Shh, N-terminal palmitoylation is facilitated by the product (Hhat) of the *hedgehog acyltransferase* gene. I have carried out a thorough analysis of Hhat in PANC1 cells. First, I characterised an antibody prepared in the lab to hHhat. I confirmed the specificity of the antibody by immunoblotting using a self-constructed hHhat-EGFP clone, and a control mGup1-EGFP clone. By subcellular fractionation and Western blotting I found Hhat to be a membrane protein. In addition, I used the hHhat antibody to determine the intracellular localisation of hHhat in PANC1 cells by confocal microscopy and showed that hHhat localised in ER mainly but not in Golgi apparatus. I confirmed this using the hHhat-EGFP clone for fluorescence microscopy in transfected cells.

To illuminate the biological function of palmitoylation of hShh in production of active hShh and in the formation of hShh multimeric complex I optimised hHhat RNAi knockdown (KD) in PANC1 cells and confirmed this by a cell-based palmitoylation assay. Using semi-quantitative RT-PCR and immunoblot analyses, I showed that hHhat KD caused decreased signalling through the Shh pathway due to reduced production of active hShh. In addition, I investigated the effect of the addition of palmitate to hShh on its association with cells by comparing hHhat KD cells with control cells. Immunoblotting suggests that palmitoylation of Shh improves its ability to associate to cell membranes. Using hHhat KD, gel filtration of high molecular weight complexes of hShh and immunoblotting of hShh I characterised the role of palmitoylation of hShh in multimeric complex formation. Lastly, I investigated the effect of hHhat KD on PANC1 proliferation and invasion, showing that it represses PANC1 proliferation and invasion.

These studies provide a firm basis for understanding the functional roles of hShh palmitoylation and its interactions with HSPGs, and provide proof-of-principle for targeting these aspects of hShh biology in tumour cell therapeutics, specifically in the pancreatic carcinoma context.

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LIST OF ABBREVIATIONS

A

ACBP	acyl-CoA binding protein
AP	alkaline phosphatase

B

BCC	basal cell carcinoma
BCNS	basal-cell nevus syndrome
Bmp	Bone morphogenetic protein
<i>botv/Extl3</i>	<i>brother-of-tout-velu</i>
bp	base pairs
BSA	bovine serum albumin

C

CD	circular dichroism
CFSE	Carboxyfluorescein succinimidyl ester
Ci	Cubitus interruptus
CML	chronic myelogenous leukemia
CNS	central nervous system
CS	chondroitin sulfate
CuAAC	Cu(II)-catalysed azido-alkyne cycloaddition
CV	Column volume

D

Disp	Dispatched
Dlp	Dally-like protein
DMEM	Dulbecco's modified Eagle's medium

E

ECM	extracellular matrix
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum

F

Fas	fatty acids
FACoA	fatty acyl coenzyme A
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
5FU	5-Fluorouracil
Fu	Fused

G

GAG	glycosaminoglycan
GF	growth factor
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GOAT	ghrelin O-acyltransferase
GPC3	glypican-3
GPI	Glycosylphosphatidylinositol

GST	glutathione S-transferase
-----	---------------------------

H

H	human
---	-------

HA	hemagglutinin
----	---------------

HEK293a	Human embryonic kidney 293a
---------	-----------------------------

Hhat	Hedgehog acyltransferase
------	--------------------------

HS	heparan sulfate
----	-----------------

HSPG	heparan sulfate proteoglycan
------	------------------------------

I

IdoA	L-iduronic acid
------	-----------------

IP	immunoprecipitation
----	---------------------

IPTG	Isopropyl β -D-1-thiogalactopyranoside
------	--

K

K14	Keratin 14
-----	------------

KD	knockdown
----	-----------

KDa	kilodaltons
-----	-------------

M

MCS	Multiple Cloning Site
-----	-----------------------

MBOAT	membrane-bound O-acyltransferases
-------	-----------------------------------

MFI	mean fluorescence intensity
-----	-----------------------------

MM	multiple myeloma
----	------------------

P

Pal-CoA	Palmitoyl-Coenzyme A
PANC1	Human pancreatic carcinoma 1
PAT	palmitoyl acyltransferase
PB	phosphate buffer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PE	phosphatidylethanolamine
PDI	protein disulfide isomerase
PIP2	phosphatidylinositol 4,5-bisphosphate
PMCC	Pearson product-moment correlation coefficient
Porc	Porcupine
Ptc	Patched
Ptc1	Patched 1
Ptc2	Patched 2

R

rEK	recombinant Enterokinase
RND	resistance nodulation-division
RT-PCR	reverse transcription-polymerase chain reaction

S

SCLC	small cell lung cancer
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel

	electrophoresis
SFM	serum-free medium
Shh	Sonic hedgehog
Smo	Smoothened
<i>sotv/Ext2</i>	<i>sister-of-tout-velu</i>
SSP	secreted signalling proteins
SuFu	Suppressor of Fused

T

T	time
TCA	trichloroacetic acid
TF	transcription factor
TGF- α	transforming growth factor- α
TMD	transmembrane domain
<i>ttv/Ext1</i>	<i>tout-velu</i>

W

Wg	Wingless
WIF	Wnt Inhibitory Factor
WT	wild-type

Y

YFP	Yellow Fluorescent Protein
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CHAPTER 1

1. General Introduction

1-1. Hedgehog (Hh) proteins and Hedgehog signalling

1-1-1. Hh proteins

Overview of Hhs. Hedgehog proteins (Hhs), acting as morphogens, can spread from producing cells to specify a diverse range of cell fates from segmental patterns in the cuticle of the *Drosophila* larva to neurons in the vertebrate neural tube, in a concentration-dependent manner [1, 2]. The Hh signalling pathway was first identified in the 1980s in a large *Drosophila* screen for genes that were required for patterning of the early embryo, originally discovered through the *Drosophila* segmental pattern mutation *hedgehog*. In mammals, all three members of Hh family proteins, Sonic (Shh), Indian (Ihh), and Desert (Dhh) Hedgehog, display a variety of roles in embryonic development, adult homeostasis, and cancer [3, 4, 5]. Although the term “Hh” strictly only applies to *Drosophila* I use the term “Hhs” here for simplicity to also encompass vertebrate hedgehog proteins, unless the distinction is crucial.

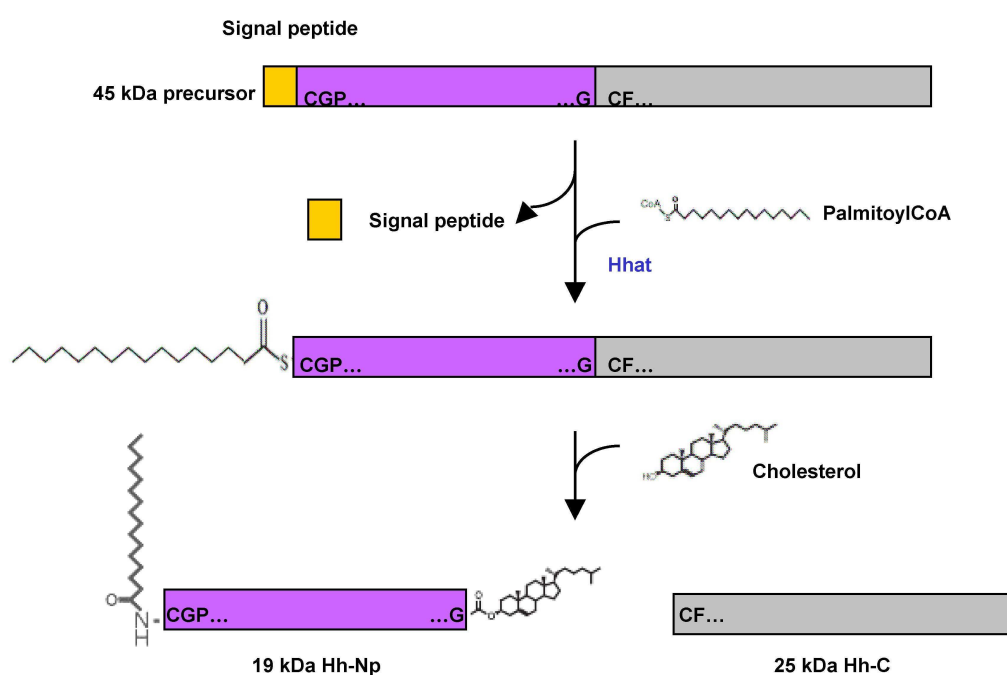
To date, the Hh signalling pathway is one of the most critical signalling pathways in both vertebrates and invertebrates [6, 7]. Perturbations to the Hh signalling pathway manifest themselves in disease; for instance, over-activity of the pathway can lead to oncogenesis and lower activity of the pathway can

result in developmental malformations [8, 9]. During differentiation and tumorigenesis, diverse targets of Hh signalling are involved in cell adhesion, signal transduction, cell cycle, apoptosis and angiogenesis [10] (Section 1-1-4, *The significance of Hh/Shh pathway and Signalling components that cause cancer*). In addition, it has been estimated that 25% of all human tumours require Hh signalling to maintain tumour cell viability. Therefore, to establish potent Hh inhibitors and biomarkers are significant goals for diagnosis and treatment of diverse human tumours.

Post-translational modifications of Hhs. The most atypical feature of Hh proteins is their post-translational modifications (Fig. 1-1), including autocatalytic cleavage, the unique N-terminal palmitoylation and C-terminal cholesterol attachment [11]. Hhs are the best established examples of cholesteroylated proteins in nature. In *Drosophila*, the approximately 45 kDa Hh precursor has its signal sequence (SS, from a.a.1 to a.a.23), removed co-translationally. It is now believed that Hhs are then palmitoylated on their highly conserved N-terminal Cys residue in the endoplasmic reticulum (ER) or Golgi complex [12]. A ~19 kDa N-terminal fragment (Hh-N) and a ~25 kDa C-terminal fragment (Hh-C) are subsequently yielded by autocatalytic cleavage catalysed by Hh-C [11]. Concurrently, a cholesterol molecule is covalently attached to the C-terminus of Hh-N thus forming the mature form of Hh, Hh-Np [14]. Unlike Hh-C, Hh-N contains all the signalling functions.

There is evidence that the role of N-terminal acylation of Hh-N is to enhance

the affinity of Hh to biological membranes and to regulate the distribution of the Hh signal [6, 14]. In addition, according to mammalian studies, cholesterol covalently attached to Hh might improve target biological activity by facilitating the interaction between Hh and its receptor Patched (Ptc) [16]. Hence, these lipid modifications are significant for Hh intracellular trafficking and to its extracellular concentration regulation.



(from *Mol. Mem. Biol.* 2009. 26(1-2): 104-113)

Figure 1-1. Post-translational modifications of Hh. The *Hedgehog* gene is expressed as a 45 kDa precursor that undergoes both dual lipidation as well as internal autoprocessing at a conserved sequence. After its signal peptide (orange region) is removed co-translationally, the precursor is palmitoylated by adding a Palmitoyl-CoenzymeA (Pal-CoA) to the new N-terminal cysteine. This N-palmitoylation is facilitated by Hhat and it has been suggested that an

intramolecular S-to-N shift occurs, transferring thioester linkage to amide linkage. Then, there is an autocatalytic cleavage at the GCF sequence catalysed by the C-terminal domain of the precursor (gray region) to produce a 19 kDa signalling domain Hh-N and a 25 kDa Hh-C. During the autocleavage event, Hh-N is modified by cholesterol at its carboxy-terminal glycine to form a fully active Hh-Np. Unlike Hh-C, Hh-Np contains all known signalling activities.

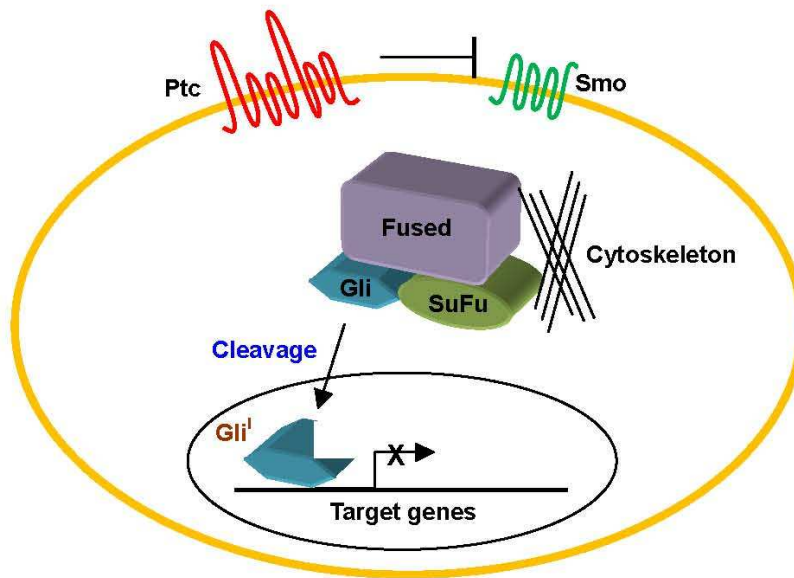
1-1-2. Hh signalling pathway components

Overview of Hh signalling. Interestingly, Hh signalling is mediated by a series of inhibitory steps. For release from producing cells, cholesterol-modified Hhs require the activity of another multispanning membrane protein Dispatched (Disp) (13,15) and hence this protein is required for long range Hh/Shh signalling. Disp, a 12-pass transmembrane protein, is related to the resistance-nodulation division (RND) family of bacterial proton-driven pumps, since mutations disturbing conserved residues important for the function of bacterial transporters prevent Shh release [17]. Disp also contains a sterol-sensing domain which may play a role in binding cholesterol attached to Hhs. Interactions with heparan sulfate proteoglycans (HSPGs) are likely involved as well (Section 1-2-1, *HSPGs in cell-cell signalling* and Section 1-2-2). The N-terminal end of Shh contains a Cardin-Weintraub sequence responsible for the binding of the morphogen to HSPGs and mutations in this domain are linked to a decrease in the proliferative activity induced by ShhN on cerebellar granule cell precursors [18]. After secretion, the diffusion of all

three Hh ligands is limited by binding to the glycoprotein Hedgehog-interacting protein (Hhip), Patched 1 (Ptc1) and Patched 2 (Ptc2) transmembrane receptors, all of which are expressed on Hh-responsive cells.

In Hh-receiving cells, Hh mediates its action via two transmembrane proteins: Patched (Ptc) displaying a transporter-like structure and Smoothed (Smo) a putative member of the G protein-coupled receptor superfamily [219] (Fig. 1-2). Ptc, a 12-transmembrane protein, is the receptor for Hh through its 2 large extracellular loops. On the other hand, Smo, a 7-transmembrane protein, is a positive transducer of Hh signalling, and it is believed that Ptc directly inhibits its biological activity. Although the exact details of ligand-receptor signalling are still under debate, the current model proposes that in the absence of ligands, Smo is impeded from signalling by Ptc. On the contrary, in the presence of active Hh, Hh binds to Ptc and this releases Smo to activate downstream signalling [19] (Section 1-1-4, *The significance of Hh/Shh pathway*). As a consequence, Smo becomes active and leads to a complex signalling cascade involving the transcription factors of the Gli family - the vertebrate homologues of the *Drosophila* Cubitus interruptus (Ci) [28]. Interestingly, the organisation of the transmembrane domains of Ptc is similar to several cholesterol-binding proteins. This suggests that Ptc is not only a cholesterol-binding protein, but also a potential key to restrain unmodified Hh from interfering with signalling [20, 21].

A. Absence of Hh



B. Presence of Hh

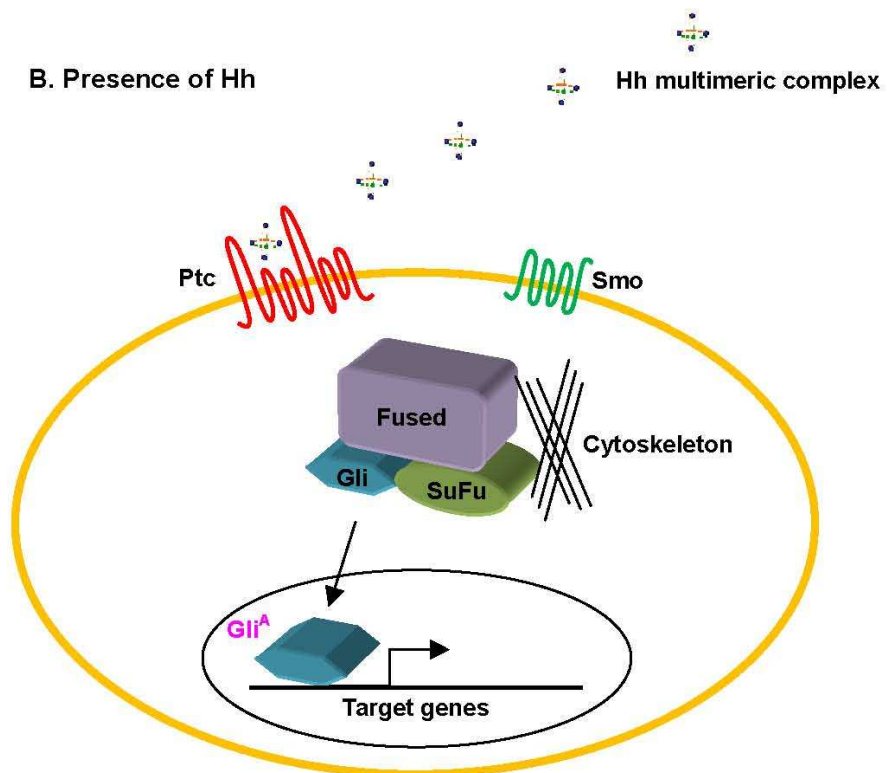


Figure 1-2. One model for how Hh multimers affect signalling in opposite directions. (A) In the absence of the ligand Hh, the receptor Ptc maintains

Smo in a compartment where it cannot engage with the downstream signalling machinery. Because of this, the Gli-modulating Fused-SuFu-Gli complex remains associated with the cytoskeleton (black bar). Gli is then cleaved, releasing the inactive form (Gli^l) that enters the nucleus and represses Hh target genes. (B) In *Drosophila*, internalisation of the Hh–Ptc complex promotes the activation of Smo. As a result, full length Gli is released from the Fused-SuFu-Gli complex and enters the nucleus to activate transcription, and subsequently stimulate the signalling activity.

Downstream signalling. Three vertebrate *Gli* genes — *Gli1*, *Gli2* and *Gli3* — have been identified. These proteins contain five zinc-finger DNA-binding domains and differ by their N-terminal domains [22,23,24]. Gli1 is considered as a means to amplify the Hh response rather than a direct effector of the Hh transduction machinery. However, Gli1 is a target gene of the pathway and is classically used as a convenient readout for the pathway activation. Gli2 would function mainly as a transcriptional activator, but also could display a repressor activity in specific contexts. Conversely, Gli3 mainly functions as a transcriptional repressor even though recent studies have also shown that Gli3 can act as an activator *in vivo* [22,23,24]. Gli proteins are post-translationally modified, and cleavage of the whole proteins results in N-terminally-truncated activator and C-terminally-truncated repressor fragments. The details of Gli activation remain obscure. However, evidence indicates that in the absence of ligands, Gli proteins are linked to the cytoskeleton by interaction with a

multiprotein complex that includes Fused (Fu) and Suppressor of Fused (SuFu). SuFu, a negative regulator of Shh signalling, interacts with the three vertebrate Gli proteins to retain them in the cytosol [178, 179] and also to control Gli function at nuclear level [25,26]. Following ligand binding, Gli proteins translocate into the nucleus where they control transcription of target genes. It is important to note that several inhibitors of the pathway are encoded by transcriptional target genes, including *Ptc*, *Gli1*, *Gli2*, *Gli3* and *Hhip* [24,27]. Hence, ligand-induced activation creates a negative feedback loop that restricts the extent of Hh signalling. As a consequence, Hh signalling is regulated at different levels by components of the pathway — a peculiar phenomenon that indicates that tight control of its activity is crucial for proper function.

1-1-3. Membrane-bound O-acyltransferase (MBOAT) family

Overview of MBOAT family. Members of the membrane-bound O-acyltransferases (MBOAT, Fig. 4-1) family are multispanning transmembrane proteins that usually catalyse the addition of a fatty acid to a hydroxyl group, typically of membrane-associated substrates such as lipids [29]. Studies based on protein purification and molecular characterisation are limited due to the presence of multiple transmembrane domains in MBOAT family proteins raising difficulties in reconstitution *in vitro*. Recent genetic screens for patterning mutations in *Drosophila* have supported the idea of enzyme-catalysed acylation by identifying a single, novel gene - *Hedgehog*

Acyltransferase (Hhat) that is required for production of an active Hedgehog signal *in vivo* [30-33]. Molecular characterisation of this gene revealed that it encodes a protein belonging to the MBOAT family of enzymes; the Wingless (Wg)/Wnt pathway gene *porcupine (porc)* is also a member of this family. Reports suggested that Porc is responsible for the palmitoylation of Wg, a morphogen involved in embryonic patterning in *Drosophila*, and its human homologues Wnts. Besides Hhat and Porc, only one other MBOAT protein, ghrelin O-acyltransferase (GOAT) has been identified in transferring fatty acids to target proteins or peptides. GOAT has been identified in metabolic activation of the 28-residue peptide hormone ghrelin by specifically acylating ghrelin on its critical serine-3 residue with medium chain fatty acids (FAs) [61,64].

Study of Hhat. The Hh family of secreted signalling proteins has been studied in a wide array of metazoan organisms. During post-translational modification of Hhs, N-palmitoylation occurs in the amino-terminal signalling domain of both *Drosophila* Hh and human Shh via amide linkage. This N-terminal palmitate is added to a highly conserved cysteine in a CGPGP motif exposed by signal peptide cleavage. It has been suggested that S-acylation of the cysteine sulphydryl could initially occur followed by a rapid and efficient intramolecular S- to N-acyl shift [34] and this is still a plausible mechanism of the N-terminal acylation. N-terminal palmitoylation of Hhs is facilitated by the product (Hhat) of the *hedgehog acyltransferase* gene (also known as *skinny hedgehog*, *sightless*, *central missing* or *rasp*) [31,33,35]. This multi-spanning transmembrane acyltransferase is directly and specifically required for the

N-terminal addition of palmitate to Hhs. Hhat has recently been definitively shown by Buglino and Resh to be a specific acyltransferase for Shh using an *in vitro* assay with purified components [12]. The reaction is clearly enzymatic and requires a free N-terminal cysteine and Pal-CoA as cosubstrate, although the concentration of Pal-CoA used is much higher than that found in cells. This could be explained by the presence of acylCoA binding protein (ACBP) in cells which may present Pal-CoA to Hhat [36]. In *Drosophila*, RNAi-mediated Hhat knockdown reduces N-terminal acylation of Hh *in vivo* and causes a reduction in cell association as indicated by a significant increase in release of Hh protein into the culture medium [31]. This study has implications not only for Hh generation and packaging but also in the distribution and reception of the Hh signalling.

Porcupine (Porc). In addition, Hhat shares homology with Porc in *Drosophila* and its *Caenorhabditis elegans* homologue Mom-1, and with GOAT, putative acyltransferases that are also part of the MBOAT family and are responsible for the palmitoylation of Wg, a morphogen involved in embryonic patterning in *Drosophila*, and its human homologues Wnts, and of ghrelin, respectively. This homology includes the conserved histidine residue that may be involved in the active site of the putative acyltransferase (Fig. 4-1). Both the Hh and Wnt pathways have emerged as playing an important role in a growing number of types of cancer [42–44] in which a general feature is unregulated activation of these pathways. On the other hand, both signalling pathways are emerging as playing homeostatic roles in the maintenance of postembryonic tissues (e.g.

stem cell maintenance in particular tissues [37–41] and possible participation in tissue repair in response to injury [42]).

As shown for Hhat, Porc is required in Wg/Wnt-producing cells for generating the fully functional signalling [45,46]. Early reports suggested that Porc is required for Wnt protein secretion, and others have demonstrated that the activity influences the N-linked glycosylation of the protein [47,48]. By metabolic labeling and mass spectrometric-based mapping analyses, Willert *et al.* [49] reported that both vertebrate and *Drosophila* Wg/Wnt proteins are modified by palmitate at a conserved cysteine residue. Mutation of this cysteine causes a loss of palmitate incorporation, and this mutation as well as enzymatic removal of the palmitate both reduce the biological activity of the protein, suggesting the function of Wg/Wnt acylation is related to interactions with receptor, membranes and lipoprotein particles (e.g. palmitoylation of Wnt5a is required for binding to its receptor Fz5 and activation of intracellular signalling [50]). It has been shown that almost all targets of the MBOAT family appear to be dually acylated in the lumen of the secretory pathway [51,52]. The most N-terminal cysteine residue after the signal sequence (e.g. Cys77 in murine Wnt3a and Cys93 in *Drosophila* Wg) is usually S-acylated [53]. Recently, a second acylated residue has been identified as Ser209 in murine Wnt3a [54] which is O-esterified with the monounsaturated fatty acid palmitoleic acid (C16:1). Interestingly, acylation of Ser209 is required for secretion of Wnt3a and possibly for Cys77 acylation, but Ser209 acylation is not dependent on acylation of Cys77. Also, for *Drosophila* Wg, Cys93 acylation

is essential for signalling activity and transport to the cell surface whereas Ser293 acylation seems to be less important for secretion but is still required for maximal signalling activity [52], implying the crucial role of membrane association. Since cysteine acylation is dependent on previous serine acylation [52,55] it is possible that Porc is responsible for the serine acylation but that the cysteine acylation is subsequently performed by a different enzyme. In that case, serine acylation would be permissive for cysteine acylation. It is important to characterise the biochemical function of Porc, as multiple Wnts play key roles in tumour formation and maintenance and human Porc itself has been found to be mutated in developmental disorders such as Focal Dermal Hypoplasia [56,57]. Hence, Porc is a potential therapeutic target in several human diseases. Interestingly, acylation is not a universal modification in the Wg/Wnt family e.g. *Drosophila* WntD has very recently been shown not to be acylated [58], in contradiction to an earlier report from the same laboratory [53]. Although WntD contains the conserved N-terminal Cys residue it does not contain an equivalent Ser209 residue, again suggesting that cysteine acylation is dependent on prior serine acylation. Finally, the dual acylation of Wg/Wnt proteins may also facilitate their interaction with membrane lipid microdomains/lipid rafts that could be the site of assembly of the lipoprotein transport packages [59], hence the dependence of Wg long-range secretion on the raft-resident protein reggie-1/flotillin-2 [60].

GOAT. Another MBOAT family member - ghrelin O-acyltransferase, GOAT - was identified and characterised simultaneously in 2008 by two independent

groups [61,62]. Ghrelin, like many other peptide hormones, is generated from a precursor protein - preproghrelin contains 117 amino acids [63, 65]. After removing the signal sequence by cleavage at amino acid-23, the proghrelin peptide sequence (94 amino acids) has an N-terminal glycine residue. The prohormone convertase PC1/3 then cleaves proghrelin after arginine-28, generating the mature 28-amino acid ghrelin peptide [66]. During posttranslational modification of the ghrelin precursor protein, serine-3 is acylated with an eight carbon fatty acid (octanoate). Octanoylation of ghrelin is a specific modification that is required for ghrelin to bind to its receptor GHS-R1a and to exert most of its biological properties. The enzyme responsible for the octanoylation of ghrelin is ghrelin O-acyltransferase (GOAT) [67,68] that is also named membrane bound O-acyltransferase 4 (MBOAT4). The activity of GOAT is dependent on its Asn and His putative catalytic residues typical of the MBOAT family. GOAT knockout and GOAT transgenic mice have been generated [69] and are indeed important tools for understanding the physiological relevance of GOAT. In 2008, Yang *et al.* published work involving an *in vitro* biochemical assay and INS-1 cells to test the sequence requirements for substrate recognition by GOAT [63]. By using mutagenesis, they demonstrated that the N-terminal glycine-1, serine-3, and phenylalanine-4 of ghrelin are crucial for GOAT recognition [63]. These three amino acids in the ghrelin sequence are absolutely conserved in all vertebrates [65]. An analogue acylated in amide linkage at position 3, [Dap³] octanoyl-ghrelin (1-5)-amide, is particularly effective as an end product inhibitor. On the other hand, modification of dietary medium chain FAs can

modulate ghrelin acylation and activity *in vivo* [70], so reduction in these could suppress appetite thus having applications in obesity and type II diabetes, whereas supplementation could promote appetite and have applications in eating disorders such as anorexia, although the substantial psychological component of these disorders cannot be underestimated. Since ghrelin has metabolic, endocrine, and clinical significance, the complete regulation of the ghrelin–GOAT system is a significant mechanism involved in the pathogenesis of obesity and diabetes. Ultimately, a better understanding of GOAT regulation and especially the development of pharmacological GOAT inhibitors or activators will be crucial for understanding the true function of the GOAT–ghrelin system and its potential as a pharmacologically treatable target. In addition, these studies may provide information that is applicable to the design of inhibitors for Porc and Hhat, for example taking advantage of the highly conserved N-terminal acylation site CysGlyProGlyArgGlyPhe of mammalian Hh proteins.

As for Hhat and Porc, GOAT presumably requires as its cosubstrate a fatty acyl coenzyme A (FACoA). This creates a topological problem as FACoAs are predominantly localised in the cytoplasm and are bound to the high affinity ACBP, possibly to prevent these highly reactive thioesters from reacting nonspecifically with cell components such as proteins [36]. Thus, to react with the luminal substrates (Hhs, Wg/Wnts and proghrelin) the FACoA would need to be transported across the ER membrane. The multispanning topology of the MBOAT members could facilitate this transport in as yet unknown ways, as

suggested recently for GOAT [62] and as early as 1996 for Porc [46], and if so this could provide another therapeutic target activity.

1-1-4. Hh/Shh signalling in cancer

The significance of Hh/Shh pathway. Hh/Shh acts as a morphogen to induce distinct transcriptional programmes and cell types based upon its local concentration [1]. Hh/Shh signalling is involved in many patterning processes. The primary cilium is a non-motile microtubule-based structure that extends from membrane-docked basal bodies in most mammalian cell types. It has recently become clear that regulation of Hh signalling in mouse and zebrafish has a conserved role in ciliogenesis which is essential for Hh signalling, but not in *Drosophila* [220]. A requirement for the cilium in Hh pathway was first suggested on the basis of mutagenesis screens in mice, which showed that genes required for ciliogenesis are also required for Hh signal transduction [220].

In vertebrates, Hh/Shh signalling ensures the development of many tissue types, including hematopoietic [71,72], osteoblastic [73,74], retinal [75,76], gastrointestinal [77,78], hair [87,88], neuronal [79,80], limb [81,82] and prostatic tissue [83,84]. Among these, Shh in the zone of polarising activity coordinately expressed with FGF4 in the apical ectodermal ridge to facilitate limb-bud development [81]. Also, Shh facilitates the expression of different transcription factors (TFs) in the developing neural tube, e.g. Pax6, Nkx2.2 and

Olig2, through controlling its concentration gradient along the dorsal-ventral axis [85,86]. Furthermore, in the developing neural tube different combinations of Shh-dependent TFs induce the fates of various cell types, including motor, floor plate, and dopaminergic neurons. Based on recent findings on Gli-stimulated transcription of cyclin B1 and cyclin D1, it suggests that via regulating cyclin-dependent cell proliferation, the pathway is able to guide tissue specification as well as tissue maintenance [89,90]. Also, the pathway provides a good model to show how the same molecular machinery can lead to different fates in different developmental tissues.

Signalling components that cause cancer. Given significance of the Hh pathway in tissue patterning and cell maintenance, mutations in Hh pathway genes or events causing misregulation of the pathway are associated with certain cancers, e.g. a distinct subset of tumours in mice and human is identified by mutations in Hh signalling components. Also, inappropriate Hh/Shh signalling is frequently related to tumour initiation and maintenance, e.g. basal cell carcinomas (BCCs) and medulloblastomas are often characterised by Ptc1 inactivation or constitutive activation of Smo [91-94].

Based on the hypothesis that constitutive activation of Hh/Shh signalling induces tumorigenesis, mutations in *Shh* have been characterised in a small percentage of BCC, medulloblastoma and also in one case of breast carcinoma cells [95]. In the majority of BCCs, gene expression profiling shows increased transcription of target genes of the Shh pathway [100]. That *Shh*

acts as a dominant oncogene was shown in studies from mice and humans, in which ectopic expression of *Shh* results in BCC [95,96]. Mutations in *Ptc1* leading to constitutive activation of *Smo* were found in many BCCs. Patients with Gorlin syndrome (also known as basal-cell nevus syndrome, BCNS) have one defective *Ptc1* allele, and are characterised by a high incidence of BCCs, medulloblastomas or other developmental defects. In transgenic mice, *Shh* overexpression driven by a constitutive Keratin 14 (K14) promoter causes development of BCC-like lesions with no UV exposure [101]. Abnormal development was observed from many of the K14-*Shh* mice, including shorter limbs, excess digits and improperly formed neural tubes. Similarly, constitutively active mutations of *Smo* have been found in 10–20% of BCCs. In addition, ectopic expression of *Gli1* or *Gli2* in the skin of *Xenopus* tadpoles or mice results in tumour formation, indicating that activating most downstream components of the pathway is sufficient to initiate tumour growth [97-99].

Medulloblastoma - the most common type of brain tumour in the young population – is associated with increased *Gli1* expression in the cerebellum and with high levels of morbidity in the range of 50% even under treatment [100]. In medulloblastoma, it is often found mutations of *Smo* or *Ptc1* in neuronal cells in the cerebellum which causes constitutive activation of *Smo*. Mutations in *SuFu* have been associated with an increased risk of medulloblastoma in humans [102]. Transgenic mice with *Ptc1*^{+/-} and *p53*^{-/-} often develop medulloblastoma.

Like in the lung, Hh signalling is active during pancreas organogenesis [103] and low-level expression of *Hip1*, *Ptc1*, *Smo*, *Ihh* and *Dhh* has been detected within mature islets and cultured cell lines [104,105,106]. Maintaining active Hh signalling via a paracrine mechanism, with the cancer cells overexpressing Shh ligand and adjacent stromal cells being competent to receive the signal, has been tested in certain pancreatic cancer cell lines both *in vitro* and *in vivo*. In *in vitro* co-culture assays, the pancreatic cancer cell lines PANC-1 and ASPC-1 (which overexpress Shh) were able to activate Gli transcription in co-cultured 10T1/2 cells [107]. More importantly, implanting the human pancreatic cancer cell line HPAF-II into *Ptc1-LacZ* mice, LacZ staining indicated upregulated *Ptc1* in the stromal cells surrounding the implant, but not in the tumour tissue. Recent evidence also indicates that downregulated Hh signalling not only causes tumour formation, but also is required for tumour maintenance, as transformed cells need active Hh continuously for survival and growth. Analysis of 26 human pancreatic adenocarcinoma cell lines showed that all lines express Hh target genes, and induced apoptosis and decreased proliferation in 50% of the cell lines tested were observed during cyclopamine treatment - a naturally occurring cholesterol analogue that inhibits Smo [108]. In pancreatic ductal adenocarcinoma (PDAC), the Shh pathway is found over-activated [109]. Also, gene expression studies in PDAC precursor lesions have demonstrated high expression levels of Shh target genes, including Gli1 and *Ptc1*, and cyclin D1. Though the molecular basis of pancreatic cancer is now better understood, the development of new diagnostic approaches and efficient treatments is still poor. PDAC remains one

of the most difficult cancers to treat. In 2000, there were 217,000 new cases of pancreatic cancer and 213,000 deaths world wide and 60,139 new patients (10.4% of all digestive tract cancers) and 64,801 deaths in Europe [223]. In 2006 there were 33,730 new cases and 32,300 deaths in the USA [223].

Shh signalling also plays a role in the pathogenesis of chronic myelogenous leukemia (CML), gliomas, and multiple myeloma (MM) [110-112]. Reya and colleagues developed mice that lacked expression of Smo. When the mice were implanted with CML stem cells, the frequency of CML was reduced by nearly 50% relative to mice expressing wild-type (WT) Smo [110]. Additionally, mice expressing a constitutively activated form of Smo had a fourfold increase in CML stem cell population relative to mice expressing WT Smo. These data provide evidence that Hh inhibition impedes CML progression by affecting CML stem cells. In human glioma [113] - a central nervous system (CNS) tumour - the transcription factor *Gli1* was overexpressed. When treated with the Smo inhibitor cyclopamine, glioma stem cell growth is inhibited [111], e.g. cyclopamine-treated CD133+ glioma stem cells showed diminished cell population by almost 50% relative to mock treatment and reduced *Gli1* and *Ptch1* mRNA levels by 50% and 40%, respectively. Myeloma (MM) is a non-Gorlin cancer affecting plasma cells [112]. In tumourgenic MM cells, transcript levels of Smo, *Gli1*, and *Ptc1* were found strongly upregulated compared to control healthy cells, as well as in MM stem cells (but not for *Ptc1*). Cyclopamine treatment reduced MM stem cell population and promoted differentiation of MM stem cells into non-proliferating mature MM cells [112].

To sum up, active Shh signalling induces the maintenance and proliferation of cancer stem cells in CML, glioma, and MM.

1-2. Functions of Heparan sulphate proteoglycans (HSPGs) in Hh/Shh signalling

1-2-1. HSPGs overview

HSPGs. Intensive biochemical and genetic studies have shown that HSPGs have crucial roles in many developmental signalling systems involved in cell fate determination and multi-cellular organisms development, such as Wnt, Hh, transforming growth factor- β (TGF- β) and fibroblast growth factor (FGF) pathways [114]. HSPGs are evolutionarily highly conserved molecules and are expressed in most animal cells. HSPGs are extracellular matrix and cell surface macromolecules that consist of a core protein and one or more HS glycosaminoglycan (GAG) chains (Fig. 1-3). Based on the structure of the protein cores, these molecules can be classified into several families [115]. Syndecans and glypicans are two major cell surface HSPGs; both are integral membrane proteins. Syndecans are transmembrane proteins that contain a highly conserved short [34–38 residue] carboxy-terminal cytoplasmic domain [115], whereas glypicans are disulfide-stabilised globular proteins linked to the plasma membrane by a Glycosylphosphatidylinositol (GPI) linkage [115]. Other cell surface HSPGs such as CD44 and Betaglycan seems to have a less significant role in ligand interactions. Interestingly, while glypicans bear exclusively HS chains, syndecans are able to associate with both HS and

chondroitin sulfate (CS). Besides syndecans and glypicans, perlecan are a group of secreted HSPGs that are mainly distributed in the extracellular matrix (ECM) and, like syndecans, can have attached CS chains in some cases [116].

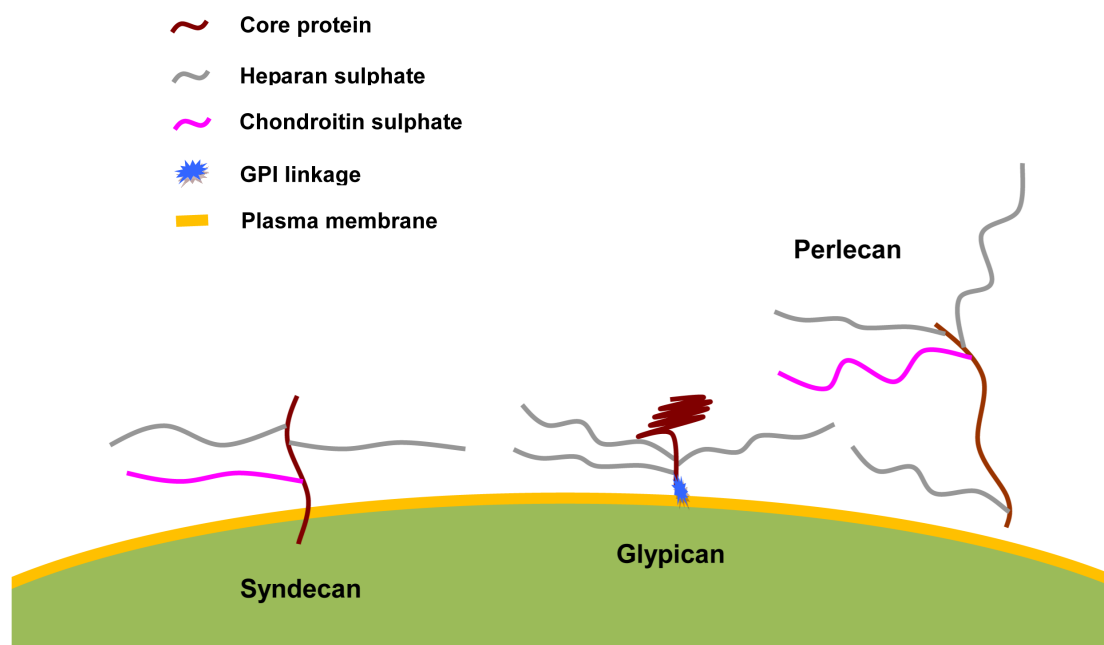


Figure 1-3. Cartoon representation of three categories of HSPGs. A syndecan with both HS and CS GAGs represents the membrane-spanning class of HSPGs. A glypican is depicted to represent the GPI-linked HSPG family. Perlecan shown carrying HS GAGs represents a group of secreted ECM HSPGs.

Biosynthesis of HS GAG chains. Both core proteins and enzymes involved in the biosynthesis of HS are conserved in vertebrates and invertebrates

[117,118]. A single syndecan gene, two glypican genes and a *perlecan* homologue have been identified in *Drosophila*. Biosynthesis of HS starts in the Golgi apparatus, at its attachment site to a core protein, which contains between 2 and 4 Ser-Gly sequences, and is further modified by sulphations and epimerisations before transport to the cell surface. Firstly, an oligosaccharide primer is attached to specific serine residues on a proteoglycan core protein. This primer serves as a substrate for the co-polymerases that add alternating linked D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) residues, resulting in repeating disaccharide chains of about 100 or more sugar units in length [115]. This polymer is subsequently modified via a series of reactions; e.g. both N-deacetylation and N- sulphation of GlcNAc units, epimerisation of GlcA to L-iduronic acid (IdoA) residues, and O-sulphation at various positions [114,115]. These additional modifications in HS chains create enormous heterogeneity in HS structure, and allow HSPGs to bind a wide variety of extracellular ligands. The proteins responsible for the addition of alternating GlcA and GlcNAc residues after initiation of the heparan chain are encoded by *tout-velu* (*ttv/Ext1*), *sister-of-tout-velu* (*sotv/Ext2*), and *brother-of-tout-velu* (*botv/Extl3*) in *Drosophila* [114].

HSPGs in cell-cell signalling. Studies supporting a role of HSPGs as co-receptors have come from genetic mutants in model organisms. Analyses of the mutants have demonstrated that HSPGs have additional roles as transducers of patterning information as well as regulators of morphogen

gradient formation. Despite the fact that a majority of these mutants have defects in cell-cell signalling, HSPGs have also been known to regulate numerous biological functions, including organisation of the cytoskeleton [127], migration [120,124], inflammation [122], metastasis [123] and synapse formation [125]. The role of HSPGs as FGF co-receptors has been well studied demonstrating an essential role of HS upstream of FGF signal transduction [126]. The model suggests that via specifically binding both the FGF ligand and its receptor to form ligands-receptor complexes, HS mediates the formation of an active transit complex and activates FGF signalling [121]. HSPGs are generally thought to function as “cell-autonomous” co-receptors on the same cell surface, but also function “cell-nonautonomously”; e.g. FGF signalling can be stimulated in cell culture by HS attached to a different cell [128]. In addition, recent studies suggest that HSPGs can have a more direct role in mediating signal transduction via cytoplasmic kinases, adapter proteins, or even the HSPG cytoplasmic domain itself to mediate signalling. For example, Syndecan-4 can recruit and activate PKC α by forming multimers in the presence of phosphatidylinositol 4,5-bisphosphate (PIP2) [129]. The PKC α recruitment seems to be an essential downstream step in FGF2 signalling in endothelial cell culture and dependent on binding both PIP2 and a PDZ-containing protein [130]. Finally, HSPGs may also present as morphogen regulators. Secreted molecules are often referred to as morphogens due to their activity in a symmetric extracellular distribution and in determining multiple cell fates across a tissue, as a consequence of their graded levels. One example is that HS mediates Hh function in embryos via regulating Hh

distribution across developing tissues [131]. In addition, analysis of loss-of-function mutations in Dally and Dally-like protein (Dlp, the *Drosophila* homologue of mammalian glypican-4/6) indicate that these molecules are involved in Hh diffusion [222], and Dlp was also shown to be needed for the reception of Hh in cultured cells and embryos of *Drosophila* [137,173]. Also, different *Drosophila* mutants suggest a role of HS and Dlp in the extracellular distribution of Wnts [132,133,134]. On the other hand, Dally has a dual role in regulating Decapentaplegic (a *Drosophila* TGF β family member) signalling, as a co-receptor and as a regulator of morphogen gradients [135].

1-2-2. Multiple roles for HSPGs in Hh/Shh signalling

The potency of HSPGs in Drosophila Hh and vertebrate Shh signalling.

Developmental and biochemical studies in *Drosophila* and vertebrate systems have proposed that HSPGs are essential for proper Hh distribution, stabilisation and signalling activity [115,136]. HSPGs are also believed to facilitate the presentation of Hh ligand to signal-receiving cells and participate in promoting cell surface microdomains/lipid rafts in which the crucial molecules are assembled into functional complexes [114]. Key pieces of evidence are as follows:

1) Flies deficient in *ttr*, *sotv* or *botv* (encoding enzymes involved in GAG biosynthesis) display embryonic phenotypes indicative of disturbed Hh, Wg/Wnt, Bone morphogenetic protein (Bmp) and FGF signalling. In the wing

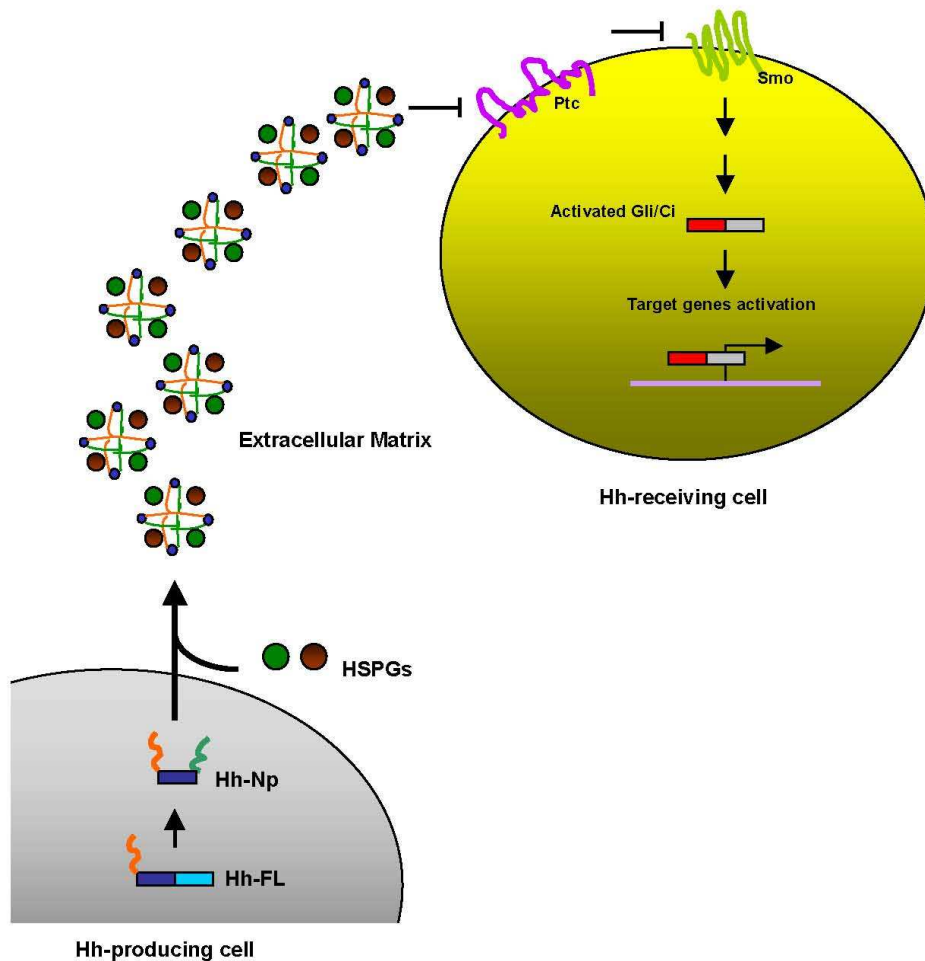
imaginal disc, Hh protein is not able to diffuse completely in *ttv*, *botv*, or *sotv* mutant clones; this phenomenon is more pronounced in *ttv-ptc* or *ttv-botv* double mutants [114]. In addition, loss of Hh protein from the ECM in *ttv*, *sotv* and *botv* clones implies a role of HSPGs in protecting Hh from degradative processes, or alternatively HSPGs may participate in releasing Hh ligand from producing cells. 2) Two core HSPG proteins in *Drosophila*, the glypicans Dally and Dlp, have been shown to be functional in the movement and reception of the Hh signal. Dally and Dlp play roles in facilitating Hh movement from its sites of synthesis [114]. In contrast, RNAi of *dlp* (but not *dally*) in clone-8 cells results in a loss of responsiveness to exogenous Hh, but this defect can be rescued by cell-autonomous expression of Hh [137]. Similarly, it has been shown in GPC3 (one of the six mammalian glypicans) null mice that expression of Hh target genes is increased in a variety of tissues, consistent with the finding that GPC3 competes with Ptc for binding with Shh at the cell surface and induces endocytosis and degradation of a Shh-GPC3 complex, removing Shh from the cell surface [138,139]. Nevertheless, GPC3 plays a positive role in Wnt signalling [140]. Conflicting data exist concerning the role of HSPGs in achieving maximal Hh signal transduction [137,141,142]. In the same studies, HSPGs could facilitate Hh ligand presentation to responding cells, concentrate Hh ligand in membrane microdomains proximal to Ptc, or may participate as part of a larger receptor complex. Despite different experimental models applied (animal versus cell culture), the data suggest that both the core protein and GAG chains contribute to reception and transduction of the Hh signal [138,139]. 3) Deficiency in the HSPG called perlecan leads to a microcephaly

in the mouse because of a decrease in cell proliferation in the medial ganglionic eminence likely related to insufficient Shh signalling [143]. 4) Mutation of Hh basic residues responsible for binding HS reduces the magnitude of Hh-induced proliferation of granule cells in culture [144], indicating that HS can act in conjunction with its core protein for stabilising or even specifying ligand-receptor complex formation.

To sum up, HSPGs seem to facilitate the efficiency of the Hh/Shh signalling cascade in *Drosophila* and vertebrate systems at various levels, including secreting, retaining and stabilising ligands, and also providing accurate trafficking and appropriate targeting to ligand-receiving cells, though this model is still unclear in human.

Cooperation between HSPGs and lipid moieties of Hh. Although the dual lipidation of Hh improves membrane affinity, other factors such as HSPGs also are required to contribute to cell surface association. More recently, it seems that only lipid-modified Hh could form into a polymeric complex [145,146] (Fig. 1-4) to enhance its solubility for long-range transportation. An *in vivo* study [146] also shows that HSPGs are required for multimers formation and might be linked to Hh by Shifted, a secreted Wnt Inhibitory Factor homologue. Using Hh immunostaining and verified by immunoprecipitation, Nicole Gorfinkiel and colleagues observed that extracellular Yellow Fluorescent protein (YFP)-Shifted colocalised with Hh at ECM and further suggested that Hh might stabilise Shifted. Also, generated *ttv* clones in a *shf*² or *shf*^{EY03173} background

leads to the suggestion that Shifted is required for the stabilisation of Hh by the HSPGs [146]. In addition, it is indicated that lipid modifications of Hh are not only essential for Hh/HSPGs interaction [145,146] but also critical for proper Ptc receptor anchoring. To sum up, the ability of lipid-modified Hhs to interact with HSPGs could influence Hh signalling and spreading efficiency through ECM. As for lipid-unmodified Hh, however, it has great difficulty to be retained and is poorly stabilised by the ECM and tends to diffuse freely [145,146].



(from *Mol. Mem. Biol.* 2009. 26(1-2): 104-113)

Figure 1-4. Model of active soluble Hh/Shh multimeric complex transport.

After post-translational modifications in Hh-producing cells, Hh-Np proteins are secreted to extracellular matrix and form a multimeric complex with HSPGs. In Hh-receiving cells, the receptor Ptc is repressed by Hh and this releases Smo to activate the downstream signalling. The presence of HSPGs can improve the solubility of Hh proteins during trafficking in extracellular matrix and enhance Hh-Ptc interaction on Hh-receiving cells as well, and therefore promote subsequent signalling activity.

1-3. Palmitoylation of Hh proteins in Hh signalling

1-3-1. Protein lipid modifications

Lipid modifications. Proteins are covalently modified with a variety of lipids, such as fatty acids, isoprenoids, GPI anchors, phosphatidylethanolamine (PE, in autophagy) and cholesterol. Lipid modifications can be divided into two groups based on their functional destinations - cytoplasmically oriented and extracellularly oriented. The former contains S-acylation (also named as thioacylation or palmitoylation), myristoylation, PE modification and prenylation (farnesylation and geranylgeranylation), while the latter includes GPI anchoring, cholesteroylation and some kinds of fatty acylation, such as of Hh proteins described herein [148]. Unlike myristoylation and farnesylation, palmitoylation alone can just provide sufficient hydrophobicity to stably anchor proteins to a lipid bilayer. N-myristoylation is the covalent addition of the fatty acid myristate to an N-terminal glycine residue via an amide linkage [149]. Prenylation is the addition of an isoprenoid (either a C15 farnesyl or a C20 geranylgeranyl group) to a C-terminal cysteine residue via a thioether linkage [150]. Lastly, palmitoylation is the covalent addition of long-chain fatty acids, usually to a cysteine residue via a thioester linkage [151-153]. On the other hand, the best-characterised lipid modification that occurs in the lumen of the secretory pathway is the attachment of GPI anchors [154]. This lipid modification is composed of a phosphatidylinositol connected through a carbohydrate linker to the protein. After the GPI moiety is added in the ER, the protein traffics through the secretory pathway and finally targets to the cell surface, where the GPI anchor embeds the protein to the extracellular face of

the plasma membrane.

The roles of protein lipidations are diverse, including the localisation and function of proteins. Among lipidations, the field of palmitoylation has advanced, in which the most common understanding for its function is to increase the affinity of a soluble protein to membranes, which can thereby regulate the protein localisation and function. Another function of palmitoylation is to regulate protein trafficking; the mechanism has been illustrated intensively for palmitoylated forms of the small GTPase Ras [152,153,155]. The third function for palmitoylation is to modulate protein stability; e.g. the yeast chitin synthase Chs3, a protein with six to eight transmembrane domains (TMDs). When palmitoylation is blocked, Chs3 aggregates and is retained in the ER, indicating palmitoylation plays an important role in a quality control mechanism for a multimembrane-spanning protein [156]. The other way for palmitoylation to adjust protein stability is by regulating protein degradation; for example, palmitoylation of the yeast sphingoid long-chain base kinase Lcb4, a soluble protein palmitoylated at two internal cysteine residues. Under normal conditions, Lcb4 is downregulated upon entry of the cells into stationary phase. On the contrary, when palmitoylation of Lcb4 is blocked by mutation this does not occur. Thus, this indicates that the half-life of the Lcb4 protein is negatively regulated by palmitoylation in normal conditions [157]. Lastly, recent studies demonstrate that lipid modifications can facilitate the efficiency and specificity of signalling through not only correctly guiding a signalling molecule to its target within the

cell but also membrane-anchoring at specific cell surface microdomains/lipid rafts [158]. For instance, the particular dual lipid modification of Hhs can influence the distribution and activity of Hh signalling proteins.

1-3-2. Multiple roles for protein lipidation in the Hh signalling pathway

The study of the N-terminal palmitoylation of Hhs. N-terminal palmitoylation of Hh is facilitated by the product (Hhat) of the *Hedgehog acyltransferase* gene (also *sightless*, *central missing* or *raspberry*). This putative transmembrane acyltransferase is classified as a member of the palmitoyl acyltransferases (PATs) for yeast and mammalian proteins [14]. Hhs are unusual in being dually lipid-modified to be fully active [159]. Moreover, it has been shown that dual lipidation is critical not only for the interactions between Hh and Ptc but also for forming a suitable complex of Hh with HSPGs to target at the Hh-receiving cell [146]. On the contrary, lipid-unmodified Hh would be released from the cell into the extracellular fluid instead of remaining in the ECM. This extracellular transmission could promote the activation of low-threshold target genes far from Hh-producing cells [145]. It is now appreciated that Ptc might be located in lipid rafts/microdomains which contains specific sets of proteins including GPI-anchored proteins, doubly acylated proteins such as Src family kinases, palmitate- and cholesterol-linked proteins [160]. Hence, the interactions between particular HSPGs, Hh and Ptc are significant to Hh spreading through the epithelium surface, as well as Hh

signal transduction.

Blocking Hhat enzymatic activity prevents formation of active palmitoylated Hhs and downregulates the Hh pathway in tumour cells which depend on active Hhs for their proliferation [161]. In the same study, the authors also confirmed the observation made previously by others that Hhat is localised in intracellular membranes of the secretory pathway, ER and/or Golgi. In cells, Pal-CoA is not free in the cytoplasm but is bound to ACBP (Section 1-1-3 and 4-1) and therefore may require a transporter that facilitates its entry into the lumen of the secretory pathway where palmitoylation of Hhs presumably occurs [162]. Another mechanism suggested by Resh [12] is that the ER lumen is a likely source of Pal-CoA for palmitoylation of Shh because free fatty acids can be specifically transported into the ER lumen, where they are converted by luminal acyltransferases into Pal-CoA.

Studies using *Drosophila* Hh variants showed that palmitoylation of Hhs is essential for effective production of the Hh signal and patterning in both wing imaginal discs and in embryos. Also, it is suggested that neither solely cholesterol modification nor N-terminal acylation of Hhs are adequate for their stable membrane localisation [7,163]. Genetic knockdown of the activity of *Hhat* by RNAi causes a reduction in overall Hedgehog protein hydrophobicity, as compared to the fully modified species, to a level that matches that of the unacylated version [31], again confirming previous propositions that the *Hhat* gene product is responsible for the N-terminal palmitoylation of Shh.

Consistent with the above observation, knockout mice deficient in Hhat are neonatal lethals and show defects in the developing neural tube and limbs similar to loss of palmitoylated Shh [164]. In the same study, overexpression of an unacylated Shh mutant (ShhC25S) in transgenic mice caused reduced activity in inducing Shh responses, and Shh protein lacking both types of lipid modification (ShhNC25S) had lower levels of residual activity.

These results support the conclusion that Hh lipidation might enable Hh to form this complex to ensure targeting to the receiving cell for efficient signalling, combined with the fact that Ptc might be located in lipid rafts/microdomains which provide platforms for signal transduction and intracellular sorting. Although this acylation, thus far, has been reconstituted *in vitro* and proved to be an enzymatic mechanism, it does not exclude the possibility that this putative acyltransferase can also act as a chaperone-like transporter *in vivo*.

The study of the C-terminal cholesteroylation of Hhs. The cholesterol moiety of Hh has been shown to associate tightly with the cell membrane [165] and is needed for Hh to be released from its producing cell under normal circumstances. The 12-pass transmembrane protein, Disp, is involved in this mechanism [13], as it is required only for the release of cholesterol-modified Hh. Thus, the cholesterol moiety of Hh is necessarily coupled to Disp to function for the proper release of Hh. Compared to fully modified Hh, a cholesterol-deficient form of Hh (HhN) has less potency to activate the Hh cascade. Moreover, HhC85S, a *Drosophila* variant that lacks palmitate due to

mutation of the acylation site, is much less potent than HhN [141] indicating that the palmitoyl adduct may play a more essential role in Hh signalling than cholesteroylation. In this study, it was also suggested that acylation plays a major role in guiding modified Hh proteins to specific membrane domains.

One model provided by Guerrero and Chiang [166] is that the cholesterol moiety increases the local concentration of Shh as well as avoiding unregulated Shh spreading and dilution. Fractionation studies of secreted Hh/Shh proteins from the supernatant of Hh/Shh-expressing cells showed that lipid-modified Hh/Shh participates in high molecular weight structures as multimeric complexes, in which cholesterol seems to mediate association [141,167,168,169,170]. Since the lipid moieties are thought to be in the core of these complexes, analogous to micelles, multimerisation of Hh/Shh might concentrate Hh/Shh locally. It has also been suggested that lipoprotein particles carry lipid-modified Hh from the cell surface, acting as vehicles for long-range transport [171]. Cholesterol might stabilise these core complexes to interact with the ECM. To sum up, the data thus far suggest that the mechanisms of Hh secretion and spreading are generally conserved throughout evolution from *Drosophila* to vertebrates, despite some functional divergences in the Hh-receiving cells. Cholesterol modification of Hh is believed essentially to play a role in restrained dilution and regulated spreading of the morphogen through the extracellular environment.

1-4. Hypothesis and aims

1-4-1. The role of HSPGs in Shh signalling

To date, Hh signal molecules are known to act as major mediators in many developmental processes and essentially require HSPGs for their proper distribution and signalling activity [172]. In addition, both the HSPG core proteins and their attached HS GAG chains are involved in this regulation [173,174]. Based on developmental and biochemical studies in *Drosophila* and vertebrate systems there are several conjectural mechanisms for how HSPGs could promote Hh signalling. On one hand, both Shh and Hh are secreted from cells as both monomeric and multimeric forms [164,175]. Soluble Hh multimeric complexes with HSPGs are freely diffusive and can improve Hh activity on target genes. On the other hand, the interaction between HSPGs and growth factors (GFs) could influence both GFs extracellular distribution and their ability to signal [126], e.g. perlecan by directly binding to Shh as a co-receptor can affect Shh signalling [176]. Furthermore, studies imply that HSPGs might participate in promoting cell surface microdomains/lipid rafts in which the plasma membrane organise into functional domains via dynamic clustering of sphingolipids and cholesterol [177]. In summary, HSPGs seem to facilitate the efficiency of these signalling cascades at various levels, including secreting, retaining and stabilising ligands, also providing accurate trafficking and appropriate targeting to ligand-receiving cells.

Studies about Shh-HSPG interactions.

In this work, I investigate the interaction between human (h) Shh and HSPGs

and clarify the biological function of hShh/HSPGs complexes in hShh signalling. The main aims are:

- To determine whether hShh-N (recombinant, commercial) binds to heparin-Sepharose (as a model of HSPG interaction), evaluate the interaction efficiency, and verify the interaction by a competitive binding test.
- If hShh-heparin binding is detected, establish an expression/purification system for wild-type and mutated hShh proteins *in vitro* via the pET-GST bacterial expression system containing mutate putative hShh residues involved in heparin binding to determine the key residues (based on modelling of Shh-heparin interaction by Dr. Barbara Mulloy, NIBSC, personal communication).
- To test biological function of purified wild-type and mutated hShhs in *C3H10T1/2* cells by determining alkaline phosphatase (AP) activity.
- To test the signalling efficacy of mutated recombinant hShhs that cannot interact with HSPGs in PANC1 cells via both Semi-quantitative RT-PCR and immunoblot analyses for the induction of Ptc and Gli1 mRNA and with Ptc/Gli1 antibodies respectively.
- To clarify the role of mutated hShhs that cannot interact with HSPGs in PANC1 cells proliferation and invasion.
- To characterise the role of mutated hShhs in Shh multimeric complex formation using Shh RNAi knockdown, followed by overexpression of wild-type and mutant Shhs, gel-filtration chromatography and Shh immunoblotting.

1-4-2. The role of hHhat in Shh signalling

To date, it has not been proved whether the palmitoylation of Shh proteins caused by endogenous Hhat occurs within the lumen of the ER, the Golgi, or both. Also, palmitoylation could occur during biosynthetic transit or at the plasma membrane. On the other hand, studies for functional roles of hShh palmitoylation and its interactions with HSPGs will provide a firm basis for understanding Shh lipidation in active Shh signalling, which provides proof-of-principle for targeting these aspects of hShh biology in tumour cell therapeutics, specifically in the pancreatic carcinoma context.

Studies about Hhat in human Shh lipidation.

The purpose of this part of my project is:

- 1) To identify the intracellular localisation of endogenous hHhat in mammalian cells.
- 2) To illuminate the biological function of palmitoylation of hShh in production of active hShh and in the formation of hShh multimeric complex. Specific aims:
 - To characterise an anti-peptide antiserum against hHhat prepared in the lab and use it to determine the intracellular localisation of Hhat by subcellular fractionation/Western blotting and immunofluorescence microscopy.
 - To establish and optimise RNAi knockdown of hHhat and test the effect on production of active palmitoylated Shh in PANC1 cells, using induction of Shh targets as a readout.
 - To clarify the role of hHhat in PANC1 cells proliferation and invasion.
 - To characterise the role of palmitoylation of hShh in Shh multimeric

complex formation using hHhat RNAi knockdown, gel-filtration chromatography and Shh immunoblotting.

CHAPTER 2

2. Materials and Methods

2-1. Cell culture

Due to the poor prognosis, rare in complete remission, and low survival rate (with fewer than 5%) of those diagnosed still alive five years after diagnosis, pancreatic cancer is a formidable disease to diagnose and treat [223]. About 95% of exocrine pancreatic cancers are adenocarcinomas, the remaining 5% include adenosquamous carcinomas, signet ring cell carcinomas, hepatoid carcinomas, colloid carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with osteoclast-like giant cells [223]. Hh signalling has a major role in the initiation and growth of pancreatic cancer [103-106]. In this study human pancreatic ductal adenocarcinoma (PDAC) PANC1 cell line was used as a model system. PDAC PANC1 cells (ATCC, CRL-1469) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum. Human embryonic kidney 293a (HEK293a) line was generously provided by Dr. Birgit Leitinger and maintained in DMEM supplemented with 8% fetal bovine serum. Due to the fact that HEK 293 cells are very easy to grow and transfect very efficiently, in this study the cells were subjected to S100/P100 fractionation and immunoblotting for the hHhat antibody characterisation purpose (Section 4-2-2). C3H10T1/2 fibroblasts generously provided by Dr. Kay Grobe were maintained in DMEM supplemented with 8% fetal bovine serum.

2-2. General cloning techniques

2-2-1. Cloning of human ShhNs-pET41a(+)

Human Shh cDNA clone was purchased from the I.M.A.G.E. Consortium (Accession No: BC111925, Clone ID: 40080739). To express and purify active Shh from *Escherichia coli*, pET41a(+)-ShhNC24II was constructed. At first, mutations were introduced into Shh by site-directed mutagenesis using the QuickChange Kit (Stratagene) to change Cys24 to Ile-Ile and add a stop codon after Gly197 from the I.M.A.G.E. clone. Then, ShhNC24II cDNA was cloned into the pET-41a(+) expression vector at the PshAI and SacI sites to form pET41a(+)-ShhNC24II. I next generated pET41a(+)-ShhNC24II/K37.38S, pET41a(+)-ShhNC24II/K178S, pET41a(+)-ShhNC24II/K37.38.178S and pET41a(+)-ShhNC24II/K74S by site-directed mutagenesis. PCR primers are self-designed using Oligonucleotide Properties Calculator and Primer X shown in Table 2-1. *E. coli* DH5 α and XL1 blue were used as competent cells for general cloning. All restriction enzymes were purchased from NEB and all PCR products were sequence-verified.

Table 2-1. PCR primers for pET41a(+)-ShhNC24II constructs

Primer	Sequence (5' to 3' direction)
C24II For	GTATGCTCGGGACTGGCGATCATCGGACCGGGCAGGGGGTTC
C24II Rev	GAACCCCCTGCCCCGGTCCGATGATCGCCAGTCCCGAGCATAC
C198X For	CAAATCGGGAGGCTGATTCCCGGGCTCGGC
C198X Rev	GCCGAGCCCGGGAATCAGCCTCCCGATTG
KK37/38SS For	GAAGAGGAGGCACCCCAGTTCCCTGACCCCTTTAGCCTAC
KK37/38SS Rev	GTAGGCTAAAGGGGTCAGGGAAGTGGGGTGCCTCCTCTTC
K178S For	GTACTACGAGTCCAGCGCACATATCCACTGC
K178S Rev	GCAGTGGATATGTGCGCTGGACTCGTAGTAC
K74S For	GAAACTCCGAGCGATTTAGCGAACTCACCCCCAATTAC
K74S Rev	GTAATTGGGGGTGAGTTCGCTAAATCGCTCGGAGTTTC
PshAI-Shh-SacI For	GATCATCGGACCGGGCAGGGGG
PshAI-Shh-SacI Rev	CGAGCTCTCAGCCTCCCGATTGTC

2-2-2. Cloning of human Shhs-pcDNA-DEST40

To establish a mammalian overexpression system of fully-lipidated hShh *in vitro*, a pcDNA-DEST40-hShh expression vector was constructed using the Gateway cloning system (Invitrogen) based on recombination between an entry vector and a destination vector. pENTR223.1-hShh (DNAFORM, provided by B Jovanović) was propagated in *E. coli* using spectinomycin (100 µg/mL) for selection while. pcDNA-DEST40 (Invitrogen) was selected by using chloramphenicol (30 µg/mL) and the for selection. pcDNA-DEST40-hShh construct by was using ampicillin (100 µg/mL). Then, pcDNA-DEST40-hShhK37.38S, pcDNA-DEST40-hShhK178S, pcDNA-DEST40-hShhK37.38.178S and pcDNA-DEST40-hShhK74S were

made using site-directed mutagenesis with primers in Table 2-1. After confirmation by sequencing and Western blot for Shh, the constructs were used for overexpression in PANC1 cells.

2-2-3. Cloning of recombinant human Hhat-pEGFP-N3

Human Hhat cDNA (Accession No: BC117130) was cloned into pEGFP-N3 mammalian expression vector at the XhoI and BamHI sites, and the mouse Gup1 cDNA (kindly provided by Yoichiro Abe) was cloned into the same vector at the same sites. The primers used in PCR are shown in Table 2-2.

Table 2-2. PCR primers for pEGFP-N3-hHhat constructs

Primer	Sequence (5' to 3' direction)
Hhat-pEGFP-N3 For	GCTCGAGATGCTGCCCCGATGGG
Hhat-pEGFP-N3 Rev	CGGATCCGTCCGTGGCGTAGGTC

2-3. Expression and purification of recombinant human ShhNs in *Escherichia coli*

The following induction and extraction protocol is based on the GE Healthcare directory. Overnight growing bacterial cultures of GST-ShhNC24II clones were diluted 1:100 into fresh pre-warmed 2xYTA medium (Tryptone 16 g/l, Yeast extract 10 g/l, NaCl 5 g/l, pH7.0), distributed into appropriate flasks and

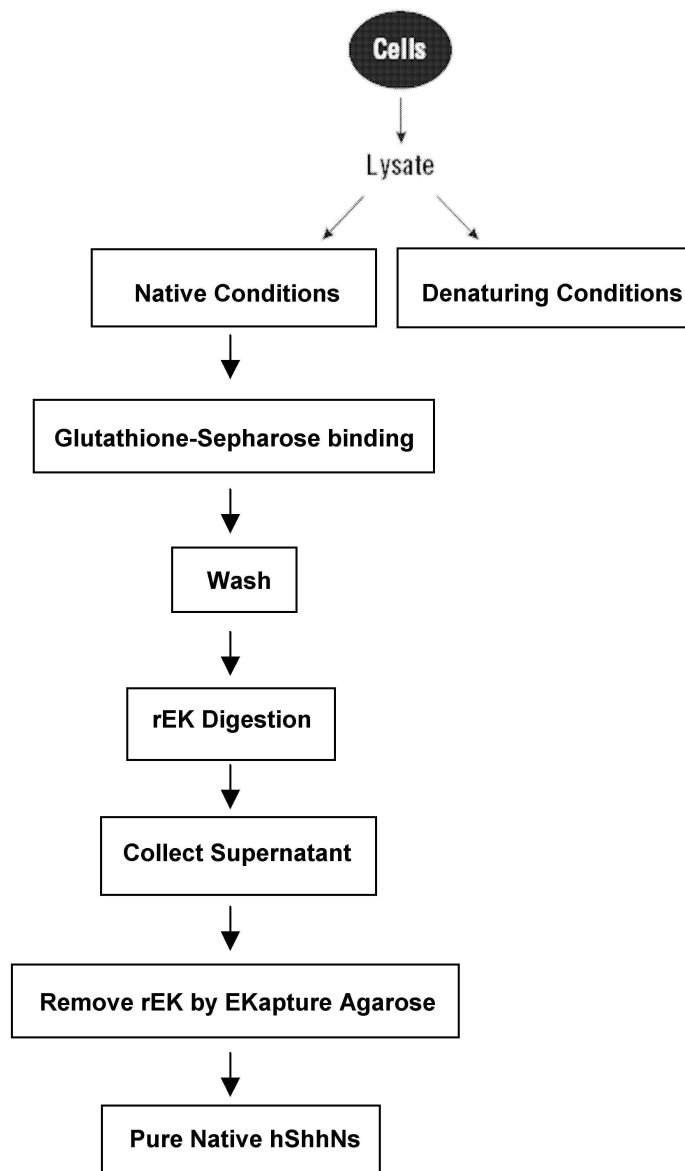
grown at 37°C with shaking until the A₆₀₀ reached 0.6. Then, 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM and incubation continued overnight at 20°C. IPTG was used as a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon. Cells were harvested (4,000 × g, 30 min) and completely suspended in 50 µl of ice-cold 1X PBS (150 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) per ml of culture. Cell lysis was done by adding 0.2% lysozyme for 30 min on ice followed by sonication (15,000, 10 sec X4, on ice) and adding 20% Triton X-100 to a final concentration of 1%. The resulting extract was centrifuged (11,000 × g, 40 min, at 4°C) to remove the cellular debris. After that, the GST-ShhC24II fusion protein was purified in a three-step procedure as described below (shown in Fig. 2-1).

Firstly, 2 ml of a 50% slurry of Glutathione-Sepharose 4B (GE Healthcare) equilibrated with 1X PBS were added to each 100 ml of bacterial sonicate. To test Glutathione-Sepharose binding efficiency, the lysates were incubated with gentle agitation at room temperature for 30 min, 2hr and 4hr and at 4°C for overnight. The Glutathione-Sepharose 4B pellet was washed with 10 column volumes (CVs) of 1X PBS for a total of three washes. 1 U recombinant Enterokinase (rEK) was subsequently added to the Glutathione-Sepharose 4B pellet directly to release ShhNC24II proteins from the slurry by cleavage of the enterokinase recognition site encoded in the vector. Incubation was done with gentle agitation at room temperature for 2hr and 4hr and at 4°C or overnight to test rEK efficiency. Lastly, residual rEK was removed by using

EKapture Agarose beads to harvest the ~19kDa soluble ShhNC24II proteins.

The rEK kit (S•Tag™ Purification Kit) was purchased from Novagen.

Figure 2-1. Diagram of the hShh-GST₂ protein purification system



2-4. Transfection and over-expression of cDNA in mammalian cells

2-4-1. Human Shhs-pcDNA-DEST40 constructs

To establish a mammalian overexpression system of fully-lipidated hShh *in vitro*, I constructed and transfected/expressed pcDNA-DEST40-hShh vector in PANC1 cells. The cloning details are described in Section 2-2-2.

For the Shh oligomerisation assay, siRNA and plasmid co-transfections were carried out sequentially in PANC1 cells (described in Section 2-14-1). Transfections of mammalian expression vectors were carried out using Lipofectamine 2000 (Invitrogen). One day before transfection, PANC1 cells were plated in 2 ml of growth medium in 6-well plates without antibiotics such that they were ~50% confluent at the time of transfection. For each transfection sample, 2.5 µg DNA were diluted in 500 µl medium without serum. After gently mixing, 2.5 µl Plus reagent were added followed by incubation for 5 min at room temperature. Then 5 µl Lipofectamine 2000 were added to the mixture and incubated at room temperature for 30 min. The DNA-Lipofectamine 2000 complexes were added to each well containing cells and medium, and mixed gently. After incubation at 37 °C in a CO₂ incubator for 48hr, the cells were ready for further analysis.

2-4-2. Human Hhat-EGFP and mouse Gup1-EGFP constructs

Transfections of mammalian expression vectors were carried out using

Lipofectamine 2000 (Invitrogen). 24hr before transfection, 1×10^5 cells were plated per well in 2 ml of the appropriate culture medium without antibiotics. For each well of cells, 2.5 µg of DNA were diluted into 500 µl medium without serum in wells of a 6-well plate. For each well of cells, 2.5 µl of Plus reagent were added to the DNA-medium mixture and incubated for 5 min at room temperature. Then, 5 µl of the Lipofectamine 2000 were added to each well containing diluted DNA, mixed gently, and incubated at room temperature for 30 min to allow DNA-Lipofectamine 2000 complexes to form. Lastly, the DNA-Lipofectamine 2000 mixture was transferred to the cultured cells. 48hr after transfection, the cells were harvested for further analysis.

2-5. RNAi knockdown

2-5-1. Human Sonic hedgehog (Shh) knockdown

hShh knockdown (KD) assay was carried out in PANC1 cells. To examine KD efficacy, immunoblotting was carried out using rabbit polyclonal anti-Shh H-160 on cells harvested at 24hr, 48hr and 72hr after siRNA transfection. Human Shh siRNA oligomers (sc-29477) were ordered from Santa Cruz Biotechnology, Inc. Negative controls that do not target any endogenous transcript are needed to control for non-specific effects on gene expression caused by introducing any siRNA. In this experiment, the negative control was SilencerR FAMTM-Labelled Negative Control #1 siRNA (Ambion). siRNA transfections were carried out with FuGENE 6 Transfection Reagent (Roche) by plating 0.3 million cells per well in a 6-well plate and 6hr later treating with

10 pmol siRNA oligomers and 3 µl FuGENE 6 Transfection Reagent. The signals from immunoblots were quantified using Scion Image software.

2-5-2. Human Hedgehog acyltransferase (hHhat) knockdown

Human Hhat siRNA duplex oligomers (Table 2-3) were designed and ordered from MWG Biotech, US. Mutated hHhat siRNA duplex oligomers (Table 2-3; Hhat-Mut) were from Invitrogen. siRNA transfections were carried out by plating 0.3 million PANC1 cells per well in a 6-well plate and 6hr later treating with 20 pmol siRNA oligomers and 3 µl FuGENE 6 Transfection Reagent (Roche) per reaction. To examine KD efficacy, immunoblotting was performed using rabbit polyclonal anti-Hhat on cells harvested at 24hr, 48hr and 72hr after siRNA transfection. The negative control for this experiment was SilencerR FAMTM-Labelled Negative Control #1 siRNA (Ambion). The signals from immunoblots were quantified using Scion Image software.

Table 2-3 Human Hhat siRNA oligomers

siRNA oligomers	Sequence (5' to 3' direction)
Hhat	UUAAUCAGGUAUGUGUACAUUCCAGUG
Hhat-Mut	UUAAUCAGGCAUAUGUACGUUCCAGUG

2-6. Semiquantitative reverse transcription — polymerase chain reaction (RT-PCR)

2-6-1. Human Shh proteins treatment of PANC1 cells

To determine the effects of different hShh proteins in Shh signalling in a human model system, I applied different purified proteins (1 µg/ml) to PANC1 cells for 24hr; after treatment with ShhNC24II, ShhNC24II/K37.38S, ShhNC24II/K178S, ShhNC24II/K37.38.178S or ShhNC24II/K74S the PANC1 cells were analysed by RT-PCR. Total RNA extraction from cultured cells was done with Trizol according to the manufacture's instruction (Invitrogen). PANC1 cells were lysed directly in a culture dish by adding 1 ml of Trizol reagent to a 3.5 cm diameter dish (the amount of Trizol reagent added is based on the area of the culture dish ~1 ml per 10 cm²), and passing the cell lysates several times through a pipette. To induce phase separation, I incubated the homogenised samples at 25° C for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added per 1 ml of Trizol reagent. Microtubes were shaken vigorously by hand for 15 sec and incubated at 25° C for 2 to 3 min. The samples were centrifuged at 12,000 × g for 15 min at 4° C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase (about 60% of the volume of Trizol reagent used for homogenisation). After transferring the aqueous phase to a fresh microtube, the RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol (0.5 ml per 1 ml of Trizol reagent used for the initial homogenisation) followed by incubating the samples at 25° C for 10 min and centrifugation at 12,000 × g for another 10 min at 4° C. After centrifugation, the RNA precipitate forms a gel-like pellet on the side and bottom of the microtube. To

wash the RNA, the supernatant was removed and the RNA pellet was washed with 75% ethanol (1 ml per 1 ml of Trizol reagent used for the initial homogenisation). The sample was mixed by vortexing and centrifuged at $7,500 \times g$ for 5 min at 4°C . At the end of the procedure, the RNA pellet was briefly air-dried for 5-10 min, then dissolved in 100 μl RNase-free water - and incubated at 60°C for 10 min. There are three quality controls that are performed on isolated RNA. One is to determine the quantity of RNA that has been isolated, the second is the purity of RNA that has been isolated and the third is the integrity of the RNA that has been isolated. Isolated RNA was quantified using UV absorption using a NanoDrop spectrophotometer and the absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A_{260}/A_{280} of 2.1 and value of 1.8-2.0 indicates that the isolated RNA is pure. After quality of isolated RNA was confirmed, the RNA was stored at -80°C ready for following reverse transcription. The integrity of isolated RNA was examined by agarous gels in Fig. 3-8A.

cDNA was synthesised by random priming from 1 μg of total RNA with SuperScript II Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions. 200 ng random primers were added to 1 μg of extracted RNA, 1 μl dNTP mix (10 mM each) and distilled water to final volume

of 13 µl in a nuclease-free microcentrifuge tube and incubated at 65° C for 5 min. After brief centrifugation, 4 µl 5X First-Strand Buffer and 2 µl 0.1 M DTT were added to each reaction and incubated at 25° C for 2 min. Then, 1 µl (200 units) of SuperScript II RT was added to each reaction followed by incubating at 25° C for 10 min and then at 42° C for 50 min. At the end of the protocol, the reaction was terminated by heating at 70° C for 15 min. The samples were now ready for PCR analysis.

The PCR primers and PCR conditions used are described in Table 2-5 [224,225]. PCR was done with KOD Hot Start DNA Polymerase (Novagen) following the manufacturer's instructions shown in Table 2-4. Cycling conditions: 29-32 cycles of 30 sec at 95°C, 30 sec at 55-60°C, and 2 min/Kb at 72°C. PCR products were resolved by electrophoresis on 1.7% agarose gels and visualised by ethidium bromide staining. The signals from RT-PCR were quantified using Scion Image software.

Table 2-4. PCR reaction setup

Component	Volumn	Final concentration
10X Buffer for KOD	5 µl	1X
25 mm MgSO ₄	3 µl	1.5 mM
dNTPs (2 mM each)	5 µl	0.2 mM (each)
PCR grade water	23.5 µl	
Sense primer (10 µM)	2.5 µl	0.3 µM
Antisense primer (10 µM)	2.5 µl	0.3 µM
Template cDNA	2.5 µl	
DNA polymerase (1 U/µl)	1 µl	0.02 U/µl
Total reaction volumn	50 µl	

Table 2-5. Primers used for RT-PCR

Gene	Sequence (5' to 3' direction)	No. cycles	Annealing temp.	Product size
<i>Shh</i>		30	60 °C	477 bp
Sense	GGCAGATATGAAGGGAAGAT			
Antisense	ACTGCTCGACCCTCATAGTG			
<i>Ptc</i>		29	60 °C	498 bp
Sense	GAAGGCGCTAATGTTCTGAC			
Antisense	TACCTAGGAGGTATGCTGTC			
<i>Gli1</i>		32	55 °C	185 bp
Sense	TTCCTACCAGAGTCCCAAGT			
Antisense	CCCTATGTGAAGCCCTATTT			
<i>Smo</i>		29	55 °C	322 bp
Sense	TTACCTTCAGCTGCCACTTCTACG			
Antisense	GCCTTGGCAATCATCTTGCTCTTC			
<i>GAPDH</i>		30	55 °C	443 bp
Sense	TTCATTGACCTCAACTACAT			
Antisense	GTGGCAGTGATGGCATGGAC			
<i>β-actin</i>		30	55 °C	1128 bp
Sense	ATGGATGAGGATATCGCTGCG			
Antisense	CTAGAAGCATTTGCGGTGCAC			

2-6-2. Human Hhat knockdown in PANC1 cells

To determine the role of Hhat in Shh signalling in a human model system, I transfected hHhat siRNA into PANC1 cells. 48hr post-transfection, the cells were harvested and processed for RT-PCR to analyse pathway activity. RNA extraction, cDNA synthesis, PCR and product analysis were done as in Section 2-6-1.

2-7. Heparin binding assay

2-7-1. Benchtop elution method

Heparin-Sepharose was purchased from Sigma (H6508). 5 µg of relevant Shh proteins (ShhNC24II, ShhNC24II/K37.38S, ShhNC24II/K178S, ShhNC24II/K37.38.178S or ShhNC24II/K74S) were applied at room temperature to a Heparin-Sepharose column equilibrated with 5-10 CVs of PBS. Then, the column was washed with 10 CVs of this buffer and eluted with a step gradient of 0.15, 0.24, 0.33, 0.5 and 0.75 M NaCl by 1 CV for each fraction. Lastly, it was washed with 5 CVs 2 M NaCl. Totally, over 20 CVs were run under gravity and each fraction was collected as 1 CV. To verify this experiment a competitive binding assay was performed as follows. 10 µg hShh proteins were mixed with free Heparin (sodium salt, Sigma, final to 1mg/ml) at room temperature for 15 min. Then this pre-incubated mixture was applied to Heparin-Sepharose following the procedures described above.

2-7-2. ÄKTA Purifier system chromatography

100 µg purified hShh proteins (ShhNC24II, ShhNC24II/K37.38S, ShhNC24II/K178S, ShhNC24II/K37.38.178S and ShhNC24II/K74S) in 100 µl phosphate buffer (described below) were applied to a Heparin affinity column (HiTrap Heparin HP, 0.7 x 2.5 cm; GE Healthcare) using an ÄKTA Purifier system (Pharmacia LKB). Elution was with a linear gradient started with 10 CVs 0 M NaCl wash, followed by 10 CVs 0-0.7 M NaCl elution, and ended with 5 CVs 0.7 M NaCl elution in 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄ (phosphate buffer, pH 7.4). As a positive control, the HepII domain of fibronectin was kindly provided by Dr. Atsuko Yoneda (University of Copenhagen) [180].

2-8. Differentiation of *C3H10T1/2* osteoblast precursor cells/alkaline phosphatase (AP) assay

1 X 10⁵ *C3H10T1/2* cells were plated per well in 6-well plates. Purified hShhNC24II proteins were added to the culture medium the day after plating at a concentration of 10, 100, 300, 500, 1000 and 2000 ng/ml. To some samples, Shh inhibitory antibody 5E1 (10 µg/ml) was added together with hShh proteins. 5 days post-hShh proteins treatment, *C3H10T1/2* cells were lysed (in 150 µl 1% Triton X-100 in PBS, pH 7.4 with protease inhibitors; complete protease inhibitor cocktail, Roche) and Shh activity was tested by measuring alkaline phosphatase (AP) induction in the cells at 405nm using a microplate reader (Bio-Rad Lumimark), accomplished by incubation with 150 µl 120mM *p*-nitrophenol phosphate (Sigma), 50 mM MgCl₂ and 1 M

diethanolamine, pH 10.4 (Sigma) at room temperature for 30 min. Assays were performed in triplicate.

2-9. Human Hhat antibody generation

In this study, in order to improve the chances of producing a successful antibodies pool, a strategy of multi-peptide co-immunisation was used in which more than one target peptides were chosen within the same protein sequences and use them as antigen in a co-immunisation protocol. Generally, having several epitopes derived from a single protein will mathematically increase the chances of obtaining a pool of antibodies that will recognise the target protein. Here, we used an anti-peptide approach to generate hHhat antibody based on two extramembrane sequences from hHhat as epitopes: KQMQQQEHD~~SL~~KASL (aa227 – aa241) and CYSHV~~G~~IAWAGTYATD (aa478 – aa493). These sequences were chosen by Prof. Magee based on prior experience, using criteria of surface exposure, hydrophilicity and antigenic potential. They were synthesised, coupled to KLH carrier and coimmunised into two rabbits by Severn Biotech Ltd., UK. Antibodies were characterised against subcellular fractions by immunoblotting.

2-10. S100/P100 fractionation

To obtain S100 (cytosol) and P100 (membrane) fractions, 0.6 million human HEK293a cells were swollen on ice for 10 min in 20 mM Tris, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 250 mM sucrose, 0.1 mM DTT containing protease

inhibitors (complete protease inhibitor cocktail, Roche), then homogenised by passing the lysates through a 23-gauge (0.6 mm) needle attached to a sterile plastic syringe for 15 times and centrifuged at low speed to remove unbroken cells and nuclei. The post-nuclear supernatants were centrifuged at 100,000 X g for 30 min at 4°C in a Beckman TLA100 rotor to yield S100 (soluble cytoplasmic material after 100,000 X g centrifugation) and P100 (membranes pelleted by 100,000 X g centrifugation).

2-11. Click chemistry/cell-based palmitoylation using bioorthogonal tag

The traditional method used for tracking protein lipid modification is based on radioisotopic labelling which is time-consuming, usually takes days, weeks or even months to process. As an alternative approach “click-chemistry” was used in this study. The term “click” was explained by Fokin to show that with the use of these methods - “Joining molecular fragments is as easy as “clicking” together the two pieces of a buckle. When its two pieces can reach each other, the buckle works no matter what is attached to it, and the components of the buckle can make a connection only with each other.” [233] One of the most common catalyst systems for Cu(II)-catalysed azide-alkyne cycloaddition (CuAAC) uses a Cu(II) salts catalyst at room temperature in the presence of a reducing agent (sodium ascorbate or metallic copper often be used). Copper catalysts (Fig. 2-2) accelerate the reaction of azides with terminal alkynes by $\sim 10^7$ relative to the uncatalysed version, making it conveniently fast at room temperature [234]. Copper and ruthenium are the

commonly used catalysts in the reaction. The use of copper as a catalyst results in the formation of 1,4-regioisomer whereas ruthenium results in formation of the 1,5- regioisomer [235]. The CuAAC reaction with a broad range of functional groups and reaction conditions has made this process have been broadly used in many applications; e.g. in polymer chemistry, medical chemistry, bioconjugations, materials science, and synthetic chemistry [236-240]. In this study, the palmitate analogue heptadec-16-ynoic acid (YnC15) was used which metabolically incorporates into proteins and then detected by following reaction with an azido-TAMRA-biotin tag.

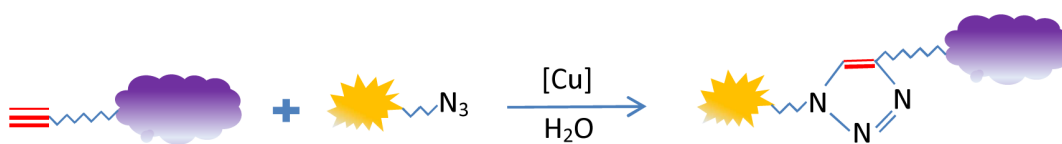


Figure 2-2. Chemical transformation of Cu(II)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. CuAAC, one of the most popular reactions within the click chemistry concept, demonstrated here used to address azides in biological systems. The reaction is very selective and efficient, but it requires addition of a copper catalyst. In 2006, Lipshutz and Taft found that copper supported on charcoal (Cu(II)/C) was an efficient catalyst [241].

48hr after siRNA transfection, the PANC1 culture medium was changed to DMEM labeling medium (1 mM sodium pyruvate and 100 μ M palmitate analogue, heptadec-16-ynoic acid YnC15, provided by Dr. Ed Tate, Chemistry Department, Imperial College) for 36hr. PANC1 cells were washed in PBS, and cell extracts were prepared in lysis buffer (1% Triton X-100 and protease inhibitors (complete protease inhibitor cocktail, Roche) in PBS, pH 7.4). Cell lysates and medium were immunoprecipitated for Shh separately using 5E1 anti-Shh MAb (Developmental Studies Hybridoma Bank, USA). Azide-alkyne cycloaddition (click chemistry [181,182]) was carried out directly on protein G-agarose beads using azido-TAMRA-biotin capture reagent. *In vitro* palmitoylation was detected by fluorescence imaging. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) following the manufacturer's suggested procedure. A standard curve for protein concentration was created here using known concentrations of bovine serum albumin (BSA) (Fig. 2-3).

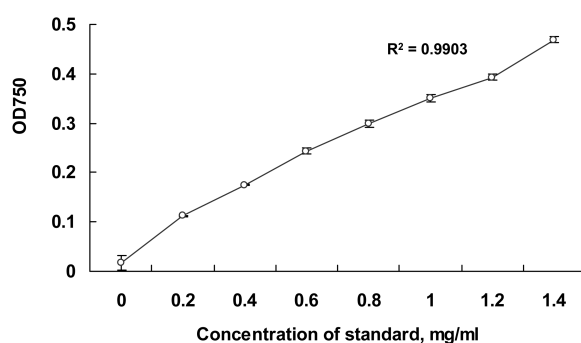


Figure 2-3. A protein standard calibration curve. Standard curve generated with BSA in water, showing concentration from 0-1.4 mg/ml. Data points are

averages for triplicates.

2-12. Western blotting

2-12-1. Human Shh proteins treatment of PANC1 cells

4hr after plating PANC1 cells (3×10^5 cells per well in a 6-well plate), 1 µg/ml purified hShh proteins (ShhNC24II, ShhNC24II/K37.38S, ShhNC24II/K178S, ShhNC24II/K37.38.178S or ShhNC24II/K74S) with or without 5E1 (10 µg/ml) were added to the cultured medium. 48hr later, cells were harvested and analysed by Western blotting.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 0.3 million cells were harvested and lysed by the addition of loading buffer (45 mM Tris (pH 6.8), 10% glycerol, 1% SDS, 20 mM DTT, 0.01% bromophenol blue) to the cell, followed by treatment with 20-30 passages through a syringe using a 23-gauge (0.6 mm) needle. After electrophoresis, proteins were transferred using a semi-dry transfer unit (AAHoefer, TE77X Semidry Blotter, USA) with 5 mA/cm² for 1hr at room temperature to nitrocellulose membranes (Millipore, Bedford, MA, USA), blocked at room temperature for 1hr in PBS with 5% skim milk, and then incubated with primary antibody diluted in PBS with 5% skim milk for 16hr at 4°C. Table 2-6 shows antibodies used in Western blotting.

Goat polyclonal anti-Patched (ab51983) and mouse monoclonal α-Tubulin (DM1A, ab49928) were purchased from Abcam. Rabbit polyclonal anti-GLI-1

(H-300, sc-20687) was purchased from Santa Cruz Biotechnology. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin IgG1, HRP-conjugated goat anti-rabbit IgG, and HRP-conjugated donkey anti-goat IgG (all used at 1:20,000; Southern Biotech). Bound immunocomplexes were detected using enhanced chemiluminescence detection reagents (Pierce) and were visualised by exposing the membrane to x-ray film (Fuji medical x-ray film, Super RX, 11860). The signals from immunoblots were quantified using Scion Image software.

Table 2-6. Antibodies used for immunoblotting analysis

Primary antibody	Description	WB application dilutions	Secondary antibody	Predicted molecular weight
H-160 (sc-9024)	Rabbit polyclonal to Shh	1:200	HRP conjugated anti-Rabbit IgG	19 kDa
5E1	Mouse monoclonal to Shh	1:10	HRP conjugated anti-Mouse IgG	19 kDa
Gli1 (H-300)	Rabbit polyclonal to Gli1	1:500	HRP conjugated anti-Rabbit IgG	117 kDa
Ptc (ab51983)	Goat polyclonal to Patched / PTCH	1:500	HRP conjugated anti-Goat IgG	150 kDa
Hhat	Rabbit polyclonal to Hhat	1:3000	HRP conjugated anti-Rabbit IgG	57 kDa

2-12-2. Hhat knockdown in PANC1 cells

For hHhat antibody characterisation, immunoblotting was carried out 24hr after transfection of HEK293 cells with appropriate expression vectors expressing hHhat-EGFP or mGup1-EGFP. For hHhat knockdown, immunoblotting was

carried out 24hr, 48hr and 72hr after siRNA transfection.

2-13. Immunofluorescence staining and confocal microscopy

PANC1 cells were seeded onto glass coverslips in 6-well plates at a density of 1×10^5 cells/well and transfected with hHhat-EGFP. 48hr after transfection, the cells were fixed with 4% formaldehyde freshly prepared in PBS at 25°C for 10 min and imaging was performed using a Zeiss LSM510 laser-scanning confocal microscope in the FILM imaging facility. Endogenous hHhat, Golgi complex and endoplasmic reticulum (ER) were visualised after staining with rabbit polyclonal anti-Hhat IgG (1:100, described above), mouse monoclonal anti-GM130 IgG1 (1:600, BD Transduction Laboratories™) and mouse monoclonal anti-protein disulfide isomerase (PDI) IgG1 (1:100, BD Transduction Laboratories™) respectively, followed by Alexa-conjugated secondary antibodies (Hhat with anti-rabbit Alexa488; GM130 and PDI with anti-mouse Alexa568).

2-14. Shh oligomerisation assay

2-14-1. Co-transfection of human Shh siRNA and pcDNA-DEST40-hShh plasmids in PANC1 cells

In this Shh oligomerisation assay, siRNA and plasmid co-transfection were carried out sequentially in PANC1 cells. I set out by plating PANC1 at 1×10^5 cells/well in a 6-well format (2 ml medium, no antibiotics). After cell attachment, cells were treated with 10 pmol of Shh siRNA following the

standard FUGENE 6 transfection protocol (described in Section 2-5-1) to knock down endogenous wild type Shh. 16hr later, pcDNA-DEST40-hShh expression vectors were transfected into the cell. The plasmid transfection protocol was described in Section 2-4-1. 72hr after the siRNA transfection, culture medium was changed to SFM for 24hr. Then, conditioned media were collected and centrifuged at 14,000 X g for 10 min. The culture media were analysed by gel filtration performed by fast protein liquid chromatography (ÄKTA Protein Purifier, Amersham Biosciences) using a Superdex200 10/300 GL column (Amersham Biosciences) equilibrated with phosphate buffer (PB; 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4) at 4. C. Elution was carried out in PB. The molecular mass standards Blue dextran, 2000 kDa; Apoferritin, 480 kDa; Alcohol dehydrogenase, 150 kDa; Albumin, 67 kDa and recombinant Human ShhN (C24II) (R&D Systems, Inc.), 19 kDa were used to calibrate the column. Eluted fractions (1 ml) were collected and were trichloroacetic acid (TCA)-precipitated (see Section 2-14-3), then examined by Shh dot blotting with antibody H-160. The signals from dot blots were quantified using Scion Image software.

2-14-2. Human Hhat KD in PANC1 cells

72hr after hHhat siRNA transfection, culture medium was changed to SFM for 24hr. Gel filtration analysis of clarified conditioned SFM was performed as above. The signals from dot blots were quantified using Scion Image software.

2-14-3. Trichloroacetic acid (TCA) precipitation

Trichloroacetic acid (TCA, also known as trichloroethanoic acid) is widely used in biochemistry for the precipitation of macromolecules such as proteins, DNA and RNA. The TCA precipitation protocol was as follows. Firstly, mix 150 μ l 100% (w/v) TCA with 1.5 ml cell culture medium and incubate on ice for 30 min. After centrifuging at 14,000 X g, 4 $^{\circ}$ C for 20 min, the pellet was washed with 1 ml 100% ice-cold acidic acetone. Repeat the centrifugation step at 14,000 X g, 4 $^{\circ}$ C for 15 min followed with air-drying for 5-10 min to remove acetone. Lastly, for SDS-PAGE add 2X or 4X sample buffer and boil sample for 10 min in 95 $^{\circ}$ C heat block before loading sample onto polyacrylamide gel.

2-15. PANC1 invasion assay

2-15-1. Human Shh proteins treatment of PANC1 cells

Biocoat Matrigel Invasion Chambers with 8 μ m pores in 6-well plates (BD Biosciences) were used in invasion assays. To determine the effect of mutated Shh proteins on PANC1 invasion ability, 72hr before PANC1 cells were plated to the chambers the cells were pretreated with hShh siRNA reagent (described in Section 2-2-1) together with exogenous recombinant Shh proteins (1 μ g/ml). The reason why 72hr was chosen here is due to reduced Shh signalling in PANC1 cells caused by pre-treated with 1 μ g/ml purified Shh proteins for 48hr (Fig. 3-8 D and E), and combined with the fact

that optimised hShh KD was observed on 72hr after siRNA transfection (Fig. 2-4). Shh proteins-containing media were changed every 48hr. PANC1 cells were detached with 5 mM EDTA in PBS, resuspended in serum-free DMEM and added to the upper compartment of the chamber (1×10^5 cells/well). Conditioned medium (8% FCS) was placed in the lower chambers. After 24hr of incubation at 37°C, the cells on the upper surface were completely removed by wiping with a cotton swab, and then the filter was fixed with 100% methanol and stained with crystal violet solution. Cells that had migrated from the upper to the lower side of the filter were photographed and counted with an inverted microscope (10 fields/filter; ID 03 Carl Zeiss Ltd, Welwyn Garden City, UK).

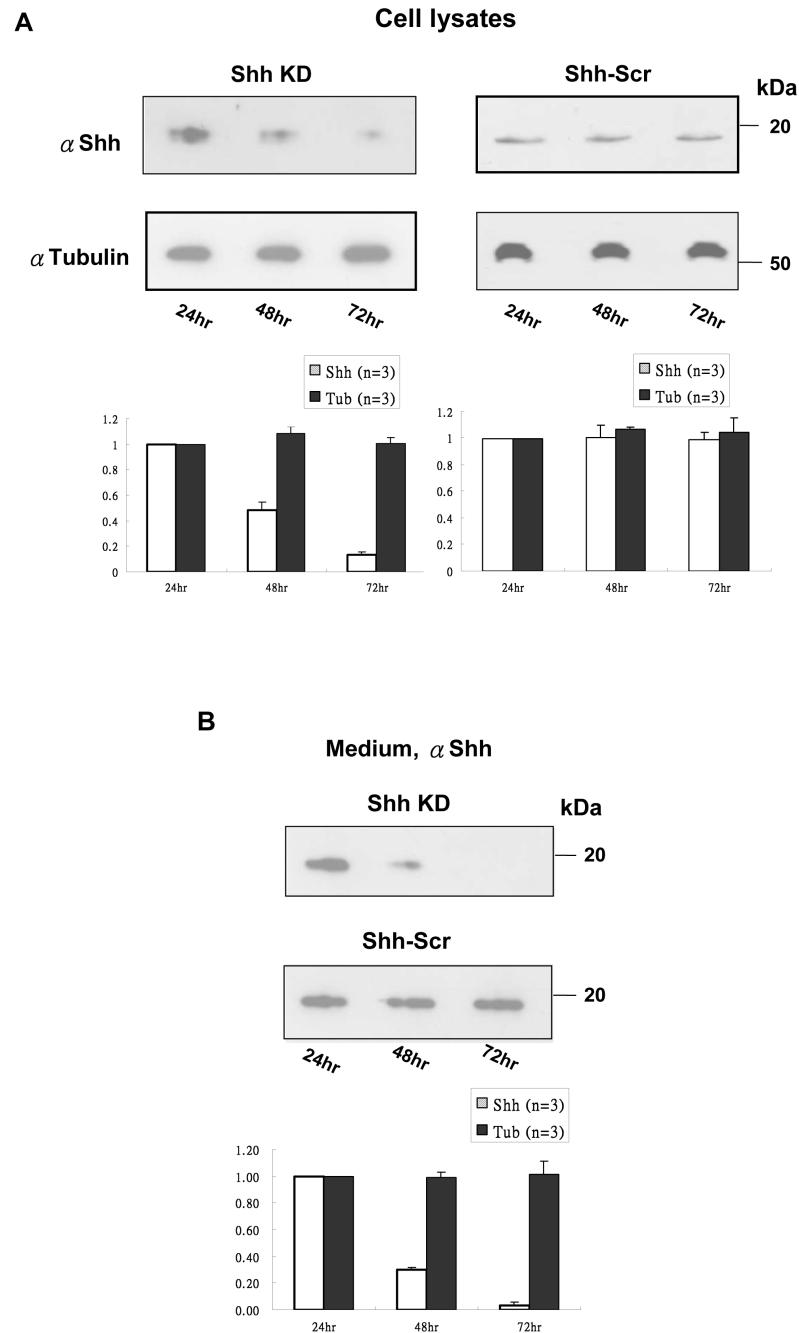


Figure 2-4. hShh RNAi knockdown in PANC1 cells. To examine hShh RNAi KD efficacy in PANC1 cells, Western blots for Shh were processed after 24hr, 48hr and 72hr siRNA transfection for both cell lysates (A) and medium (B). Tubulin was used a loading control. Shh-Scr (scrambled siRNA) was used as a negative control to specify Shh siRNA. In B, medium samples were TCA

precipitated then examined by Shh Western blots. The signals from immunoblots were quantified using Scion Image software. Correspondent quantitative results are shown below Western blots.

2-15-2. Human Hhat KD in PANC1 cells

To determine the effect of hHhat KD on invasion, PANC1 cells were pretreated with Hhat siRNA (described above) for 72hr before they were added to the Matrigel Invasion chamber and invasion assessed as above. The reason why 72hr was chosen here is due to optimised condition for reduced Shh signalling in PANC1 cells caused by Hhat KD was observed on 72hr after siRNA transfection (Fig. 4-8 C and D).

2-16. PANC1 proliferation assay and flow cytometry

2-16-1. Human Shh proteins treatment of PANC1 cells

Carboxyfluorescein succinimidyl ester (CFSE, Sigma, Cat. No. 21888), a fluorescent cell staining dye, was used in the PANC1 proliferation assay. Due to the hydrolysis of CFSE by intracellular esterases, once CFSE incorporates into cells it is not transferred to adjacent cells. Combined with the fact that CFSE will distribute to two daughter cells equally following each cell division, CFSE represents an extremely valuable fluorescent dye for proliferation study.

The detailed protocol was as follows. Briefly, after 24hr of synchronisation in SFM, cells were treated with hShh siRNA together with purified hShh proteins (1 µg/ml each; hShhNC24II, hShhNC24II/K37.38S, hShhNC24II/K178S, hShhNC24II/K37.38.178S or hShhNC24II/K74S) or 5E1 (Day1 sample, 10 µg/ml) separately, and then all samples were labeled with 2.5 µM CFSE at 37 °C for 15 min. Shh proteins-containing media were changed every 48hr. 5E1-Day4 (10 µg/ml) treatment was carried out 72hr after synchronisation, to mimic siRNA KD kinetics *in vivo*. After 8 days stimulation, cell division was indicated by decreased CFSE fluorescence intensity per cell, as analysed by flow cytometry. For flow cytometer assessment, PANC1 cells were removed from plates with ice-cold FACS buffer (1% FCS and 2 mM EDTA in PBS) and resuspended in the same buffer. Cells were analysed with a FACScalibur flow cytometer.

2-16-2. Human Hhat KD in PANC1 cells

Briefly, after 24hr of synchronisation in SFM, cells were transfected with siRNAs (test and controls) or treated with anti-Shh 5E1 blocking antibody (Day1, 10 µg/ml) and then all conditions were labeled with 2.5 µM CFSE at 37 °C for 15 min. For some wells, 5E1 treatment was carried out 72hr after synchronisation (Day4, 10 µg/ml), to mimic RNAi KD. After 8 days of culture, cell division was indicated by decreased CFSE fluorescence intensity per cell, as analysed by flow cytometry as above.

2-17. Statistical analysis

All experiments were replicated at least three times, and statistical significance was measured by using the two-tailed t test. A P value of less than 0.01 was considered to indicate statistical significance. The two-tailed test is a statistical test used in this study. This contrasts with a one-tailed test, in which only one of the rejection regions sufficiently small or sufficiently large is preselected according to the alternative hypothesis being selected, and the hypothesis is rejected only if the test statistic satisfies that criterion. Generally, if the distribution from which the samples are derived is considered to be normal, Gaussian, or bell-shaped, then the test is referred to as a one- or two-tailed T test. If the test were performed using the actual population mean and variance, rather than an estimate from a sample, it would be called a one- or two-tailed Z test.

CHAPTER 3

3. The Role of HSPGs in Human Shh Signalling

3-1. Introduction

Proteoglycans include secreted forms and cell-associated forms. Structurally, HSPGs consist of a core protein classified into three distinct classes - the Syndecans with a single transmembrane domain, the Glypicans with a GPI anchor and a varied group of secreted proteoglycans, based on their core protein structure - with one or more HS chains. Functionally, heparan sulfate proteoglycans (HSPGs) not only mediate significant interactions between cells and their environment but also regulate the distribution of extracellular signalling molecules such as morphogens through binding to them [183-186]. This great potential is based on HSPGs' enormous structural differences - partly via the additional modifications in HS chains through the repeating disaccharide chain elongation and sulphation.

To date, Hedgehog (Hh) / Sonic hedgehog (Shh) signal molecules are known to act as major mediators in many developmental process and essentially require HSPGs for their proper distribution and signalling activity [172]. Nevertheless, the mechanisms of their dependence in man are still unclear. In the case of Hh/Shh long-range signalling in *Drosophila* and mice, the activity is enhanced through forming a multimeric complex to increase Hh/Shh solubility, which is one critical criterion for protein stability [187]. Notably, this complex form demonstrates higher signalling efficiency than the monomer and the form

requires both the HSPG core proteins and their attached HS GAGs [173,174,187,188]. Based on previous studies, there are several conjectural mechanisms for how HSPGs promote this signalling, as follows. On the one hand, both Shh and Hh are secreted from cells as both monomeric and multimeric forms [164, 169]. This soluble Hh/Shh multimeric complex with specific HSPGs - Perlecan and Glypican - is freely diffusive and can regulate Shh signalling [176, 189]. On the other hand, the interaction between HSPGs and growth factors (GFs) could influence both GFs extracellular distribution and their ability to signal [190]; e.g. Perlecan by directly binding to Shh as a co-receptor can affect Shh signalling [176, 191]. More recently, the finding that only lipid-modified Hh could assemble into a polymeric complex [146] to enhance its solubility for long-range transportation might be linked to Hh by Shifted via interacting with both HSPGs and Hh proteins. Shifted is a secreted Wnt Inhibitory Factor (WIF) homologue. This study indicates that lipid modifications of Hh are not only essential for Hh/HSPGs interaction [145] but also critical for proper Ptc receptor anchoring through binding to Ptc. In contrast, lipid-unmodified Hh has great difficulty to be retained and poorly stabilised by the ECM and tends to diffuse freely [145]. Furthermore, studies implied that HSPGs might participate in promoting cell surface microdomains/lipid rafts in which the crucial molecules are assembled into functional complexes [177]. To sum up, HSPGs seem to facilitate the efficiency of these signalling cascades at various levels, including secreting, retaining and stabilising ligands, and also providing accurate trafficking and appropriate targeting to ligand-receiving cells [173, 192,193].

A Cardin-Weintraub sequence located in the N-terminal end of Shh is thought to be responsible for the binding of the morphogen to HSPGs and appropriate localisation of Hh proteins [18,206]. Nevertheless, studies provided by Chan suggested that the Cardin-Weintraub motif did not seem to be solely responsible for Shh/HSPG binding [207]. In this work, I set out to use DNA mutagenesis to determine hShh residues involved in Heparin binding. Then, using RT-PCR and immunoblot analyses of Gli1 and Ptc (Hh signalling target genes; detecting mRNA and Gli1/Ptc protein respectively) to analyse hShh signalling efficacy of mutated hShh that cannot interact with HSPGs in PANC1 cells. Since, I use human cancer cells as my model system, this study would provide evidence to shed light onto the importance of HSPGs in hShh signalling and cell transformation. Ultimately, blocking this interaction could be a therapeutic option.

3-2. Results

3-2-1. Purification of human Sonic Hedgehog (hShh) proteins bacterially expressed in the pET41a(+) system

To establish a purification system for hShh *in vitro*, pET41a(+)-hShhN was constructed as shown in Fig. 3-1. All the constructs were sequence-verified.

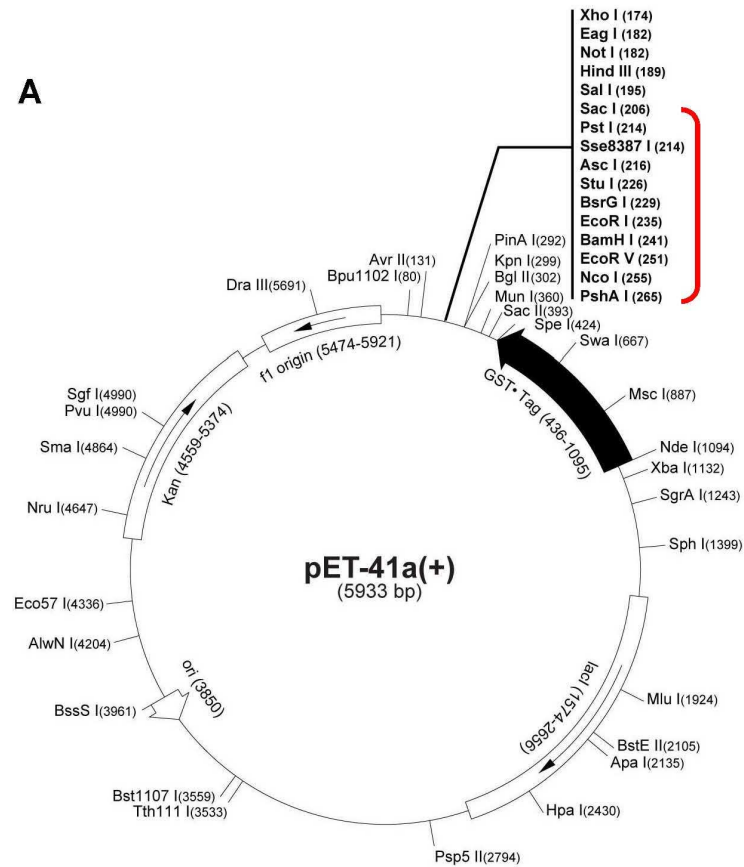
IPTG induces the transcription of the gene coding for β -galactosidase, a hydrolase enzyme that catalyses the hydrolysis of β -galactosides into monosaccharides. IPTG intake is independent on the action of lactose permease, since other transport pathways are also involved. One advantage of IPTG for *in vivo* studies is that as it cannot be metabolised by *E. coli* its concentration remains constant and the rate of expression of *lac* operon-controlled genes is not a variable in the experiment. For induction, a sterile 1 M solution of IPTG is typically added by 1:1000 dilution into a logarithmically growing bacterial culture.

In this study in order to determine whether IPTG concentration has an effect on the level of hShh-GST expression different IPTG concentrations were tested in induction assays, 0.1 mM and 1 mM (Fig. 3-2A). Both IPTG levels caused similar induction of hShhN-GST clone and the proteins all express to high levels. Next, to investigate the time required for Glutathione-Sepharose to interact with hShhN-GST fusion protein as the first purification step, a study of the time course of binding was performed over 2hr, 4hr and 6hr (Fig. 3-2B). The unbound fractions were obtained by centrifugation and analysed by

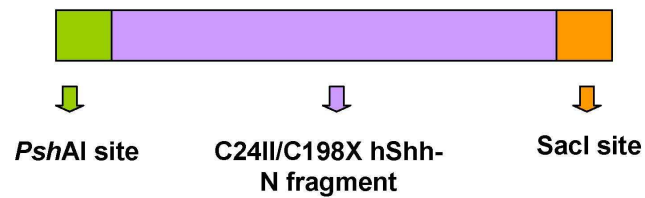
SDS/PAGE (4hr and 6hr results not shown), indicating that binding for 2hr at room temperature has reached the maximum binding capacity of the hShhN-GST fusion protein.

After the fusion proteins were isolated from a cell extract by Glutathione-Sepharose affinity chromatography, to remove hShh proteins from Glutathione-Sepharose beads efficiently, the time course of recombinant Enterokinase (rEK) digestion was carried out for 2hr, 4hr, 6hr and overnight. The analysis of cleavage products was examined by Coomassie Blue-stained SDS-PAGE gels (Fig. 3-2C, left panel; only show 4hr results). It indicated that rEK cleavage is most efficient after 4hr incubation at room temperature. Then, after hShh isolation from Glutathione-Sepharose, EKapture Agarose was used according to the manufacturer's recommendations to remove rEK from the hShh/rEK mixture. Purity of hShh proteins was assayed by Coomassie Blue-stained SDS-PAGE gels (Fig. 3-2C, right panel) and concentration of purified proteins was estimated by measuring absorbance at 280 nm using a Nanodrop spectrophotometer. Gel electrophoresis here provided showed good quality of purified proteins.

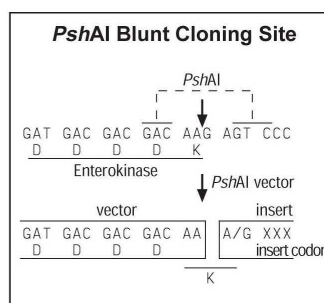
A



B



C



D

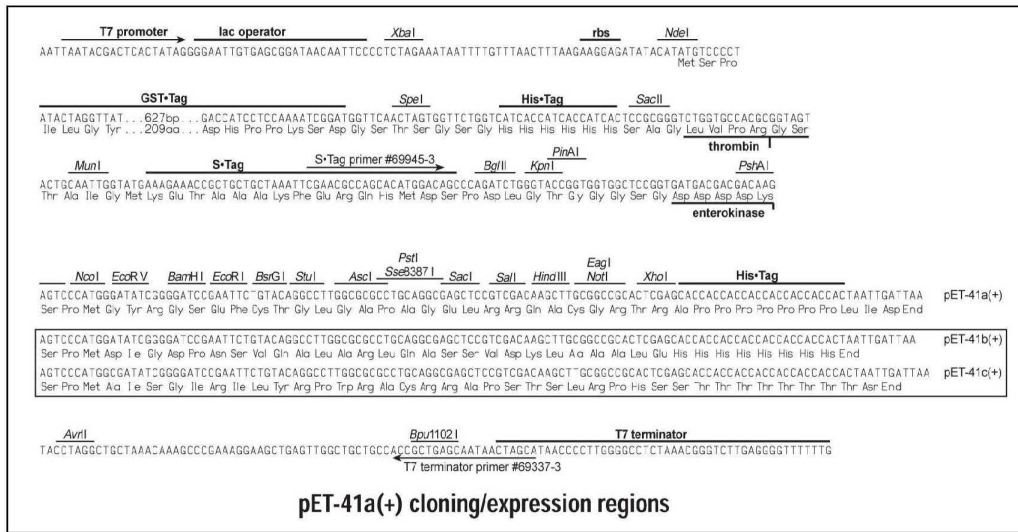


Figure 3-1. Cloning map of hShhN-GST fragments. A: pET-41a(+) vector designed for cloning and high-level expression of peptide sequences fused with the 220 aa GST•Tag™ protein. The red line indicates the insertion sites of hShh fragments. Unique sites are shown on the map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map. B: PstAI-hShhN-SacI PCR fragment used for expression/purification in bacteria. C: PstAI blunt cloning site. Vector-encoded sequence can be completely removed when cloning into the PstAI site and then cleaving the GST fusion protein with rEK. D: pET-41a(+) cloning/expression regions.

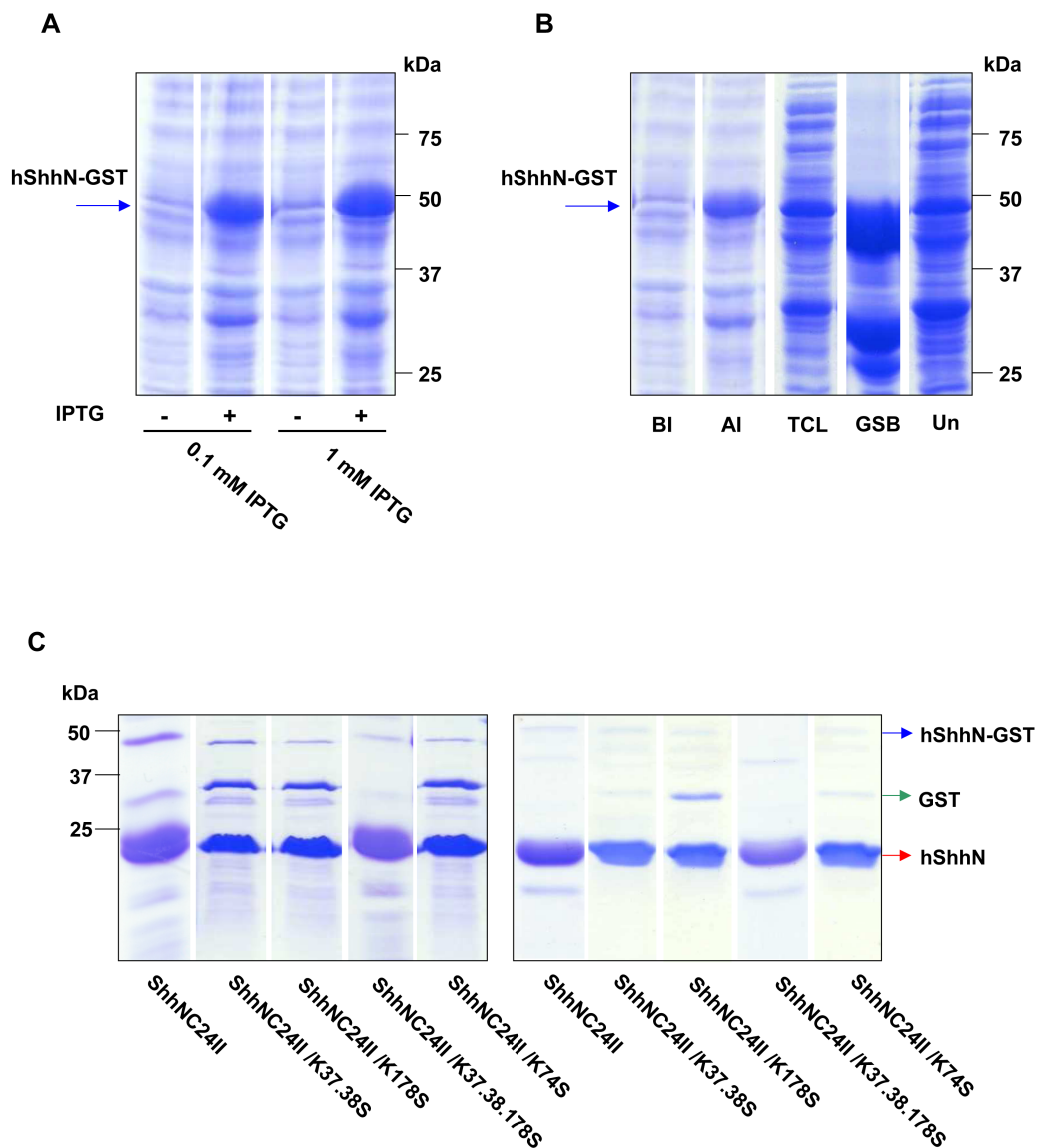


Figure 3-2. Purification of hShh proteins expressed in bacteria. A: 0.1 mM and 1 mM IPTG induction of hShhNC24II in the GST system. B: induction and purification of hShhNC24II using the GST system. BI: cell lysate before induction; AI: cell lysate after induction with 0.1 mM IPTG; TCL: total cell lysate with 0.2% lysozyme; GSB: Glutathione-Sepharose bound fraction; Un: Glutathione-Sepharose unbound fraction. Blue arrow shows predicted size of hShhN-GST fusion protein with 19 kDa hShhN and 30 kDa GST. C: analysis of rEK digestion of hShhN-GST fusion proteins. The left panel shows the

pattern of hShh proteins digested by rEK for 4hr. The right panel provides the purity of hShh proteins after removal of rEK from the hShh/rEK mixture. Red arrow shows predicted size of hShhN protein at 19 kDa. Green arrow shows predicted size of GST at 30 kDa. Blue arrow shows predicted size of undigested hShhN-GST fusion protein at 49 kDa.

3-2-2. Characterisation of purified hShh proteins via Western blots

The correct three-dimensional structure is essential to function. To examine the bioactive folding of mutated hShhN proteins purified from *E. coli*, two different hShh antibodies, 5E1 (Shh neutralising antibody) and H160, were used in Western blotting (Fig. 3-3A) and dot blotting (Fig. 3-3B). The former was used to define the native folding of purified hShh proteins [169,194,221]; the latter was used to prove equal amount of purified hShh proteins were loaded. However, to determine if protein folds into its characteristic and functional three-dimensional structure from random coil, further techniques for studying protein folding will need to be used; e.g. circular dichroism (CD), dual polarisation interferometry, X-ray crystallography and NMR. Folded structures of proteins are routinely determined by X-ray crystallography and NMR. Dynamic methods to characterise protein folding such as dual polarisation interferometry and CD provide a measurement of conformation and conformational change rather than structure.

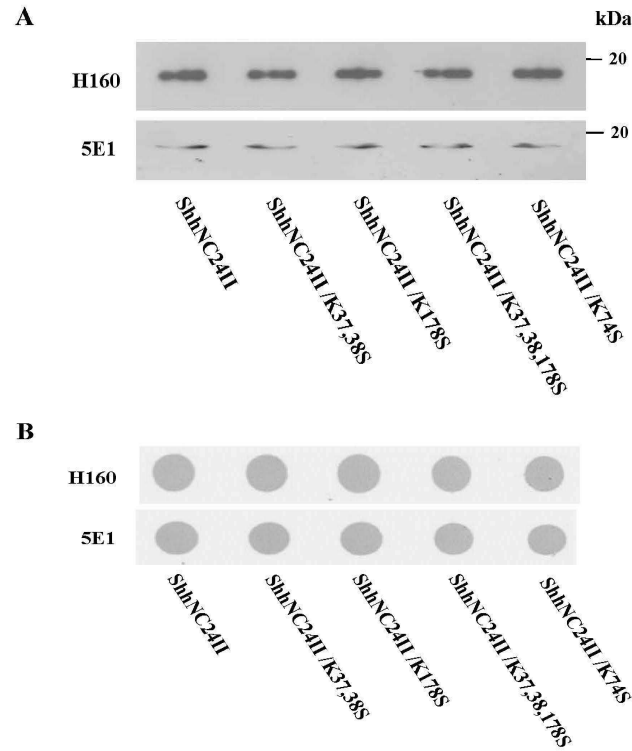


Figure 3-3. Characterisation of purified hShh proteins by immunoblotting. Purified hShh proteins were analysed by Western blot (A) and dot blot (B) with two Shh antibodies, H160 and 5E1.

3-2-3. K37/38 and K178 of hShh play crucial roles in Heparin interaction

Previous studies in *Drosophila* [115] and vertebrate systems [136] suggested that by forming a multimeric complex with specific HSPGs - Perlecan and Glypican – the efficiency of Hh signalling could be facilitated. However, this mechanism is still unclear in the human system. Putative hShh residues involved in heparin binding were based on modelling of Shh-heparin

interaction provided by Dr. Barbara Mulloy (NIBSC, personal communication; Fig. 3-4). This modelling supported the role of the known Shh Cardin-Weintraub motif in heparin interaction but suggested the involvement of another lysine residue, K178. To examine whether hShh could interact with HSPGs, I established a simple bench-top heparin affinity assay (Fig. 3-5) and verified the result in the presence of competing free heparin (data not shown). The hShh bound to heparin, albeit quite weakly, and was competed by heparin. This suggests that hShh has potential to form a complex with HSPGs, as already known.

Next, to verify previous results another system was used to determine key residues on hShh involved in HSPG binding, involving column chromatography on a heparin-Sepharose column with gradient elution (Fig. 3-6). Again, elution profiles from column chromatography together with results from gel electrophoresis provided in Fig. 3-2C (right panel) both demonstrate good quality of purified proteins. In this assay, mutated hShh proteins (ShhNC24II, ShhNC24II/K37.38S, ShhNC24II/K178S, ShhNC24II/K37.38.178S and ShhNC24II/K74S) were expressed and purified from bacteria and were applied to Heparin affinity chromatography and eluted by a 0 to 0.7 M NaCl gradient (Fig. 3-6). Fig. 3-6B and 3-6C show that ShhNC24II eluted in 0.54 M NaCl (consistent with previous binding assay data), similarly to ShhNC24II/K74S (0.53 M NaCl), whereas ShhNC24II/K37.38S (Fig. 3-6D) and ShhNC24II/K178S (Fig. 3-6E) show reduced interaction, being eluted in 0.4 M NaCl and 0.3 M NaCl fractions respectively. Very interesting to note, when I

examined the triple mutant, ShhNC24II/K37.38.178S, the elution pattern showed much lower binding efficacy, eluting at 0 - 0.06 M NaCl (n=5), suggesting that K37, K38 and K178 on hShh play critical roles in HSPG binding.

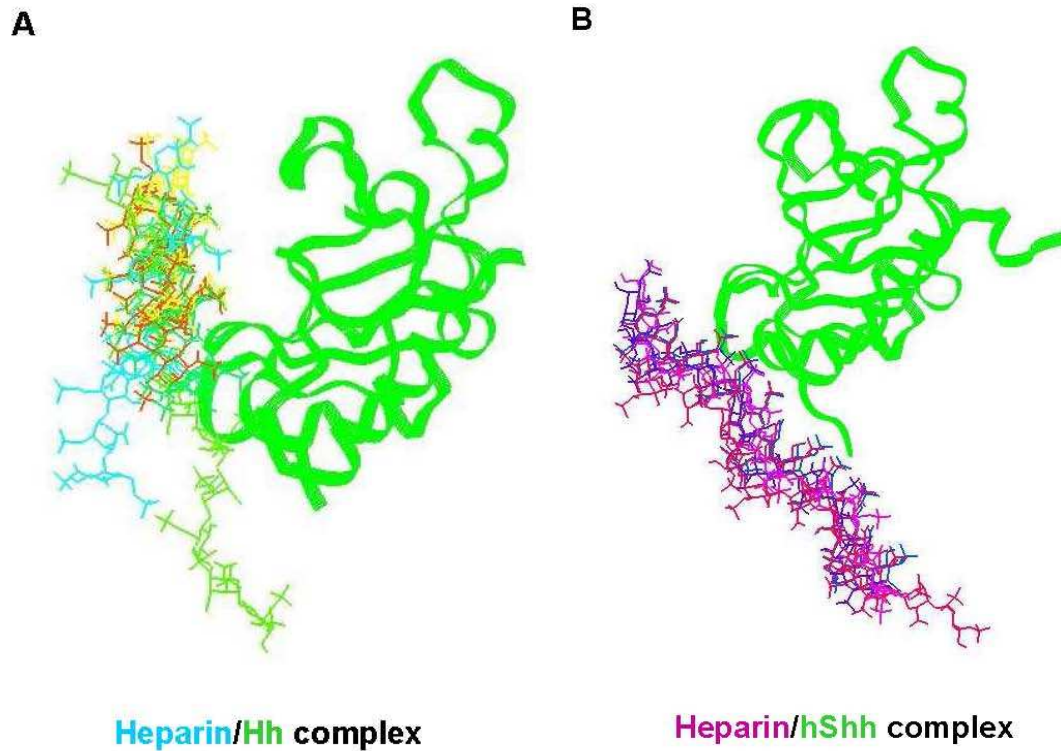


Figure 3-4. Computer modelling of heparin/Hh and heparin/hShh. The computer modelling suggests interaction between heparin to *Drosophila* Hh (A) and heparin to hShh (B). Heparin is shown as blue and purple in A and B respectively. Hh and hShh are shown as green in A and B respectively (Barbara Mulloy, personal communication).

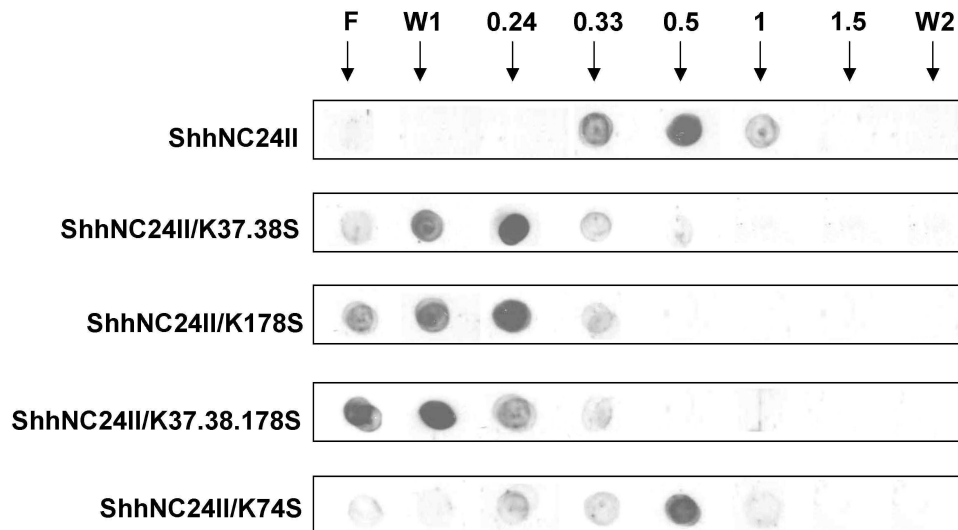
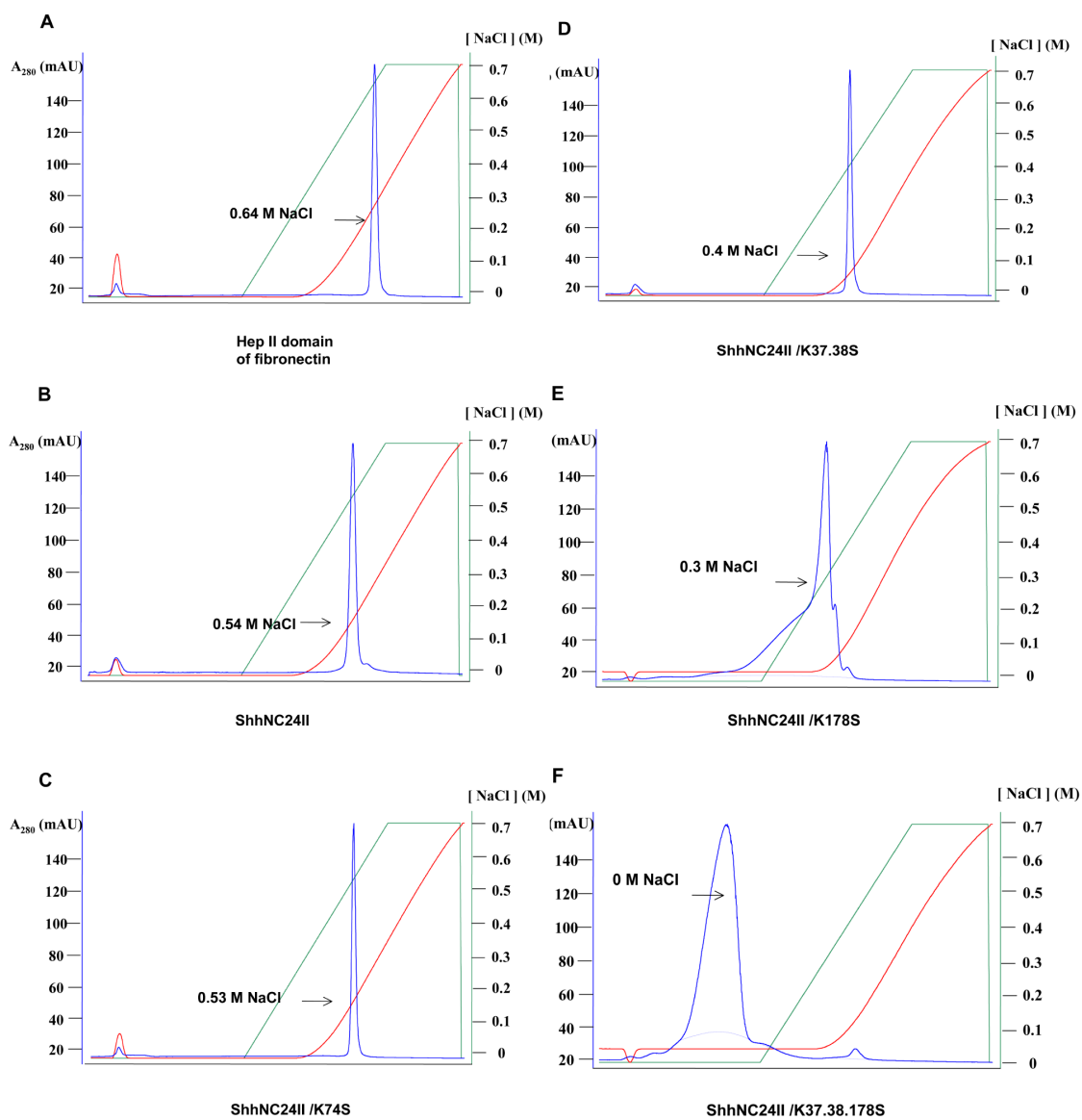


Figure 3-5. Bench-top heparin/hShh proteins binding assay. 5 μ g of purified hShhN proteins were applied at room temperature to a Heparin-Sepharose column equilibrated with 10 column volumes (CVs) of PBS. Then, the column was washed with 10 CVs of this buffer and eluted stepwise with 0.24, 0.33, 0.5, 1, 1.5 and 2 M NaCl in PBS. Totally, over 20 CVs were run at a gravity rate and each fraction was collected as 1 CV. F: 1st CV flow through; W1: Wash 1 fraction with 10 CVs 0.15 M NaCl; W2: Wash 2 fraction with 5 CVs 2 M NaCl; 0.24: eluate with 1 CV 0.24 M NaCl; 0.33: eluate with 1 CV 0.33 M NaCl; 0.5: eluate with 1 CV 0.5 M NaCl; 1: eluate with 1 CV 1 M NaCl; 1.5: eluate with 1 CV 1.5 M NaCl. After collection, 10 μ l volumes of each fraction were spotted on nitrocellulose membrane and probed with anti-Shh 5E1.



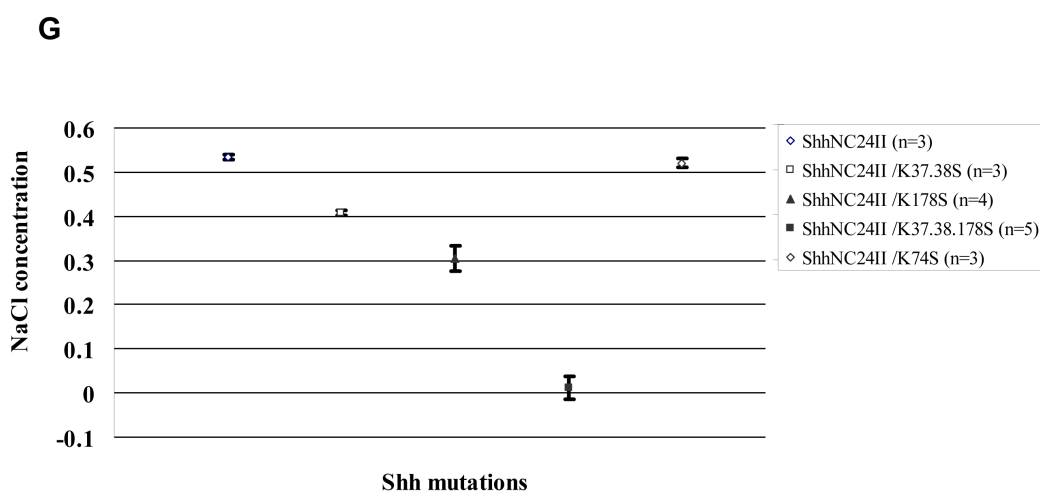


Figure 3-6. Characterisation of purified hShh proteins with heparin affinity chromatography via ÄKTA Purifier system. The individual Heparin binding efficacy was determined via Heparin-Sepharose chromatography (A-F) with a liner gradient of 0-0.7M NaCl. Relative affinities of hShh variants for heparin, represented by [NaCl] required for elution, are shown in G. In A-F, the X-axis represents time. Blue line shows OD 280 absorbance. Green line demonstrates elution gradient of 0-0.7M NaCl phosphate buffer. Red line represents the conductivity curve. Column: HiTrap Heparin HP, 0.7 x 2.5 cm; Flow Rate: 0.5 ml/min at room temperature; Buffer A: 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4; Buffer B: 0.7 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4.

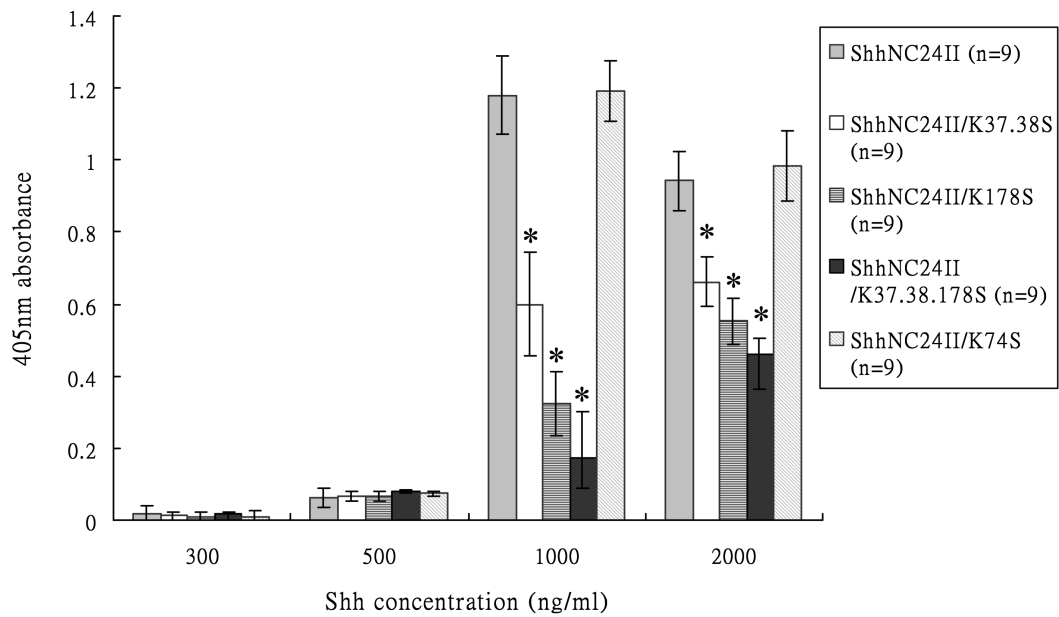
3-2-4. Bioactivity of mutated hShhs in *C3H10T1/2* cells

To examine biological function of different hShh proteins, I took advantage of *C3H10T1/2* osteoblast precursor cells which induce alkaline phosphatase (AP) when stimulated by Shh [188]. As shown in Fig. 3-7A, both ShhNC24II and ShhNC24II/K74S supplemented medium (1µg/ml) induced *C3H10T1/2* differentiation into AP-producing osteoblasts, evidenced by increased AP activity detected by 405nm absorbance due to *p*-nitrophenylphosphate substrate hydrolysis, demonstrating competent biological activity of these purified hShh proteins. In contrast, medium containing ShhNC24II/K37.38S and ShhNC24II/K178S were greatly reduced in their activity in the *C3H10T1/2* assay with 0.60 ± 0.14 and 0.32 ± 0.09 A_{405} respectively. Furthermore, ShhNC24II/K37.38.178S triple mutant exhibited a more significant loss of AP activity with only 0.17 ± 0.13 A_{405} . However, when I increased hShh proteins to 2 µg/ml the differences of AP activity between hShhs-containing medium become less significant - A_{405} ShhNC24II: 1.18 ± 0.11 ; ShhNC24II/K74S: 1.19 ± 0.08 ; ShhNC24II/K37.38S: 0.60 ± 0.14 ; ShhNC24II/K178S: 0.32 ± 0.09 and ShhNC24II/K37.38.178S: 0.17 ± 0.13 (Fig. 3-7A).

Next, to verify whether that the induced AP activity was due to hShh proteins, I used Shh-neutralising antibody 5E1 [194] as a specific Shh inhibitor during *C3H10T1/2* differentiation (Fig. 3-7B). As expected, the biological activity of Shhs was significantly inhibited by 5E1 co-treatment - A_{405} ShhNC24II: 0.41 ± 0.07 ; ShhNC24II/K74S: 0.41 ± 0.05 ; ShhNC24II/K37.38S: 0.20 ± 0.05 ; ShhNC24II/K178S: 0.15 ± 0.01 and ShhNC24II/K37.38.178S: 0.08 ± 0.01 (Fig.

3-7B). The above data showed that ShhNC24II/K37.38S, ShhNC24II/K178S and particularly ShhNC24II/K37.38.178S mutants were greatly reduced in activity both in their ability to bind Heparin and to induce AP expression, suggesting that both K37.38 and K178 are significant in Shh biological activity.

A



B

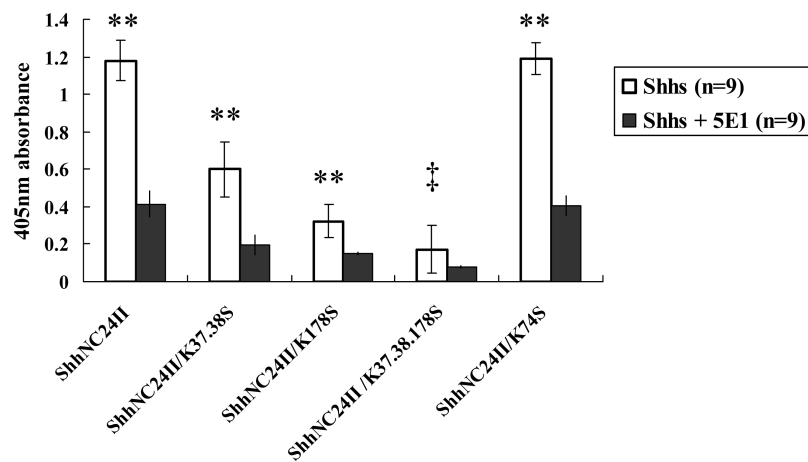


Figure 3-7. Differentiation of *C3H10T1/2* Osteoblast Precursor Cells - AP assay. *C3H10T1/2* osteoblast precursor cells were cultured in the presence of different hShh mutants from 300-2000 ng/ml for 5 days (A). The relative amount of Shh-induced AP activity was measured as described in Materials and Methods to determine biological activity of the morphogen. As a control,

Shh-treated cells were incubated with 5E1 anti-Shh to demonstrate specificity of Shh-induced AP (B). Values are mean $\pm$ S.D., n=9 in each group, **p*-value <0.001 compared to control group ShhNC24II, ***p*-value <0.001 compared to 5E1 treatment group, ‡*p*-value <0.01 compared to 5E1 treatment group.

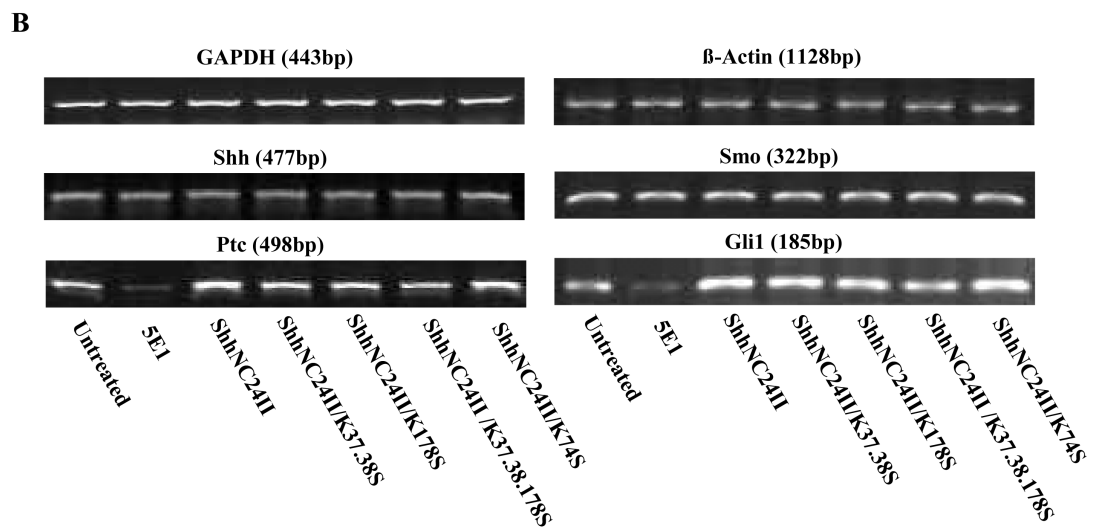
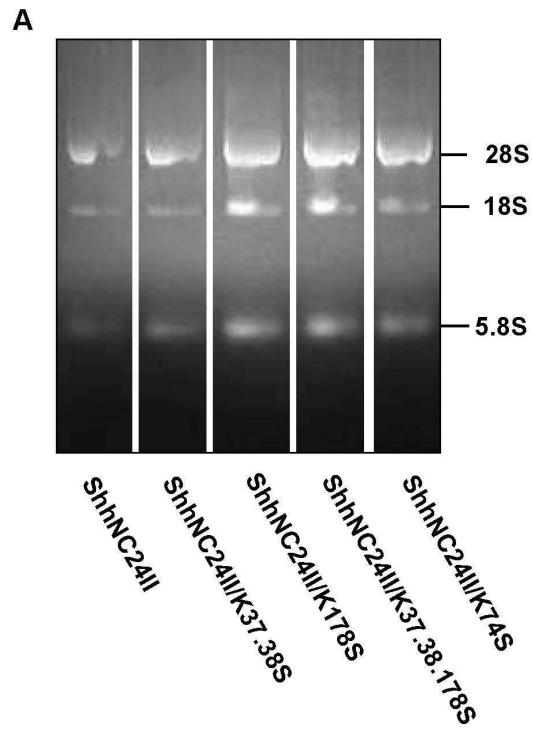
3-2-5. ShhNC24II/K37.38.178S is deficient in causing increased signalling through the Shh pathway in PANC1 cells

To provide further direct evidence that mutated hShhs were deficient in Shh signalling activity, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blots were carried out on PANC1 cells pre-treated with 1 μ g/ml hShhs for 24hr and 48hr respectively. Therefore, the signalling activity obtained from both assays will be the consequence of endogenous hShh plus exogenous purified Shh proteins in PANC1 cells.

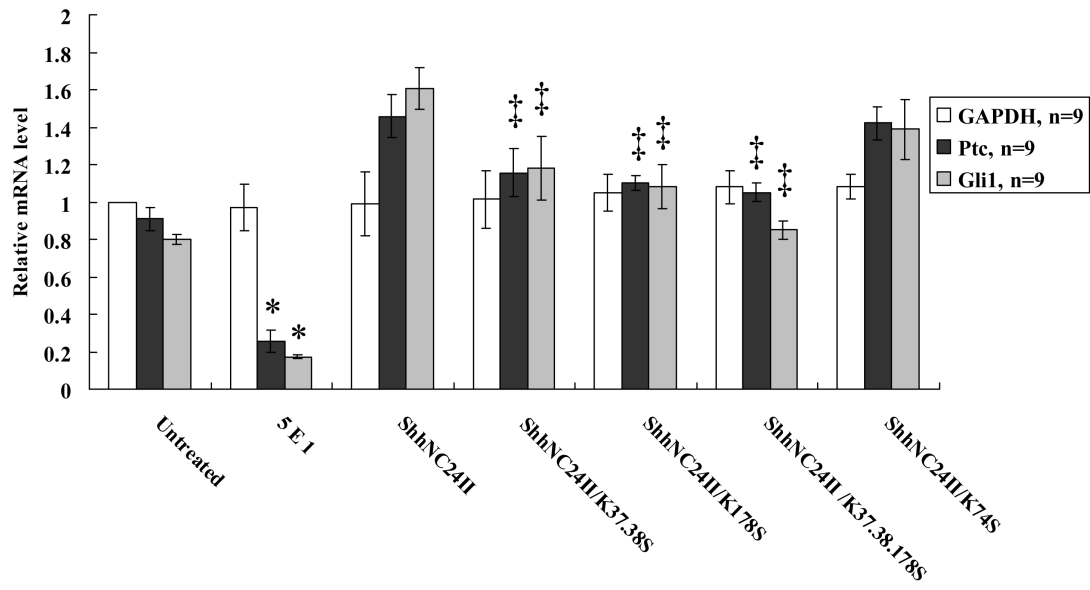
The quality of extracted total RNAs is provided in Fig. 3-8A, showing three clear distinguishable rRNA species, 28S, 18S and 5.8S. Isolated RNAs were quantified using UV absorption using a NanoDrop spectrophotometer measured at 260 and 280 nm. RNA has its absorption maximum at 260 nm and OD A_{260}/A_{280} ratio is used to assess the RNA purity of an RNA preparation. Pure RNA has an A_{260}/A_{280} of 2.1. In this study, values of A_{260}/A_{280} between 1.8-2.0 were detected in all five conditions (Fig. 3-8A) indicating good quality of isolated RNAs. In RT-PCR (Fig. 3-8B, D), GAPDH and α -Actin were used as loading controls. Ptc and Gli1 were used as indicators of Shh pathway activity,

as they are targets of this pathway. In comparison with untreated cells, 5E1 treatment strongly reduced both Ptc and Gli1 expression by 70% and 79% respectively. On the other hand, compared to ShhNC24II and ShhNC24II/K74S, both ShhNC24II/K37.38S and ShhNC24II/K178S showed decreased Ptc and Gli1 mRNA level by > 20% and > 25% respectively ($P < 0.001$), whereas Shh and Smoothed (Smo) showed no significant difference. More importantly, ShhNC24II/K37.38.178S showed a further reduction in Ptc and Gli1 by > 28% and > 47% respectively ($P < 0.001$).

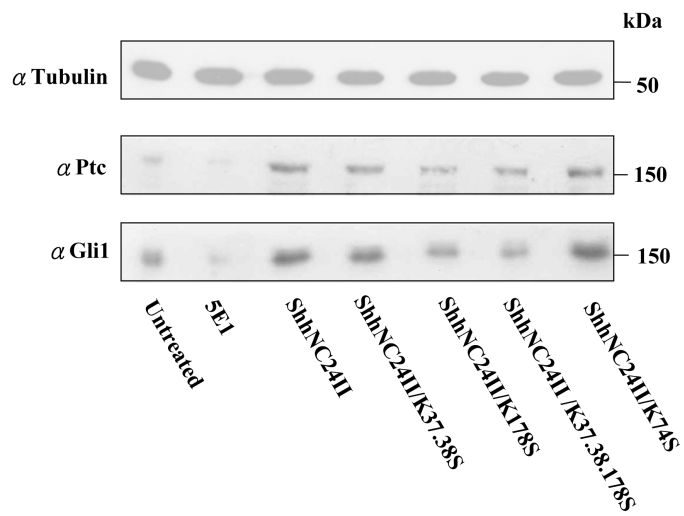
Next, immunoblotting was carried out to verify RT-PCR results (Fig. 3-8E, F). Interestingly to note that in Western blots of Ptc both ShhNC24II/K178S and ShhNC24II/K37.38.178S showed strong reduction in protein level by 44% and 60% respectively ($P < 0.0003$), comparing to ShhNC24II and ShhNC24II/K74S treated condition. On the other hand, less Gli1 be stimulated by ShhNC24II/K178S and ShhNC24II/K37.38.178S with 53% and 57% reduction respectively ($P < 0.005$) compared to ShhNC24II and ShhNC24II/K74S group. To sum up, modifications of wild type Shh through potential HSPG binding sites (K37.38 and 178) resulted in a large reduction in the activity in both *C3H10T1/2* and PANC1 assays (Fig. 3-7 and 3-8), suggesting that HSPG binding is crucial to Shh signalling in human model system.



C



D



E

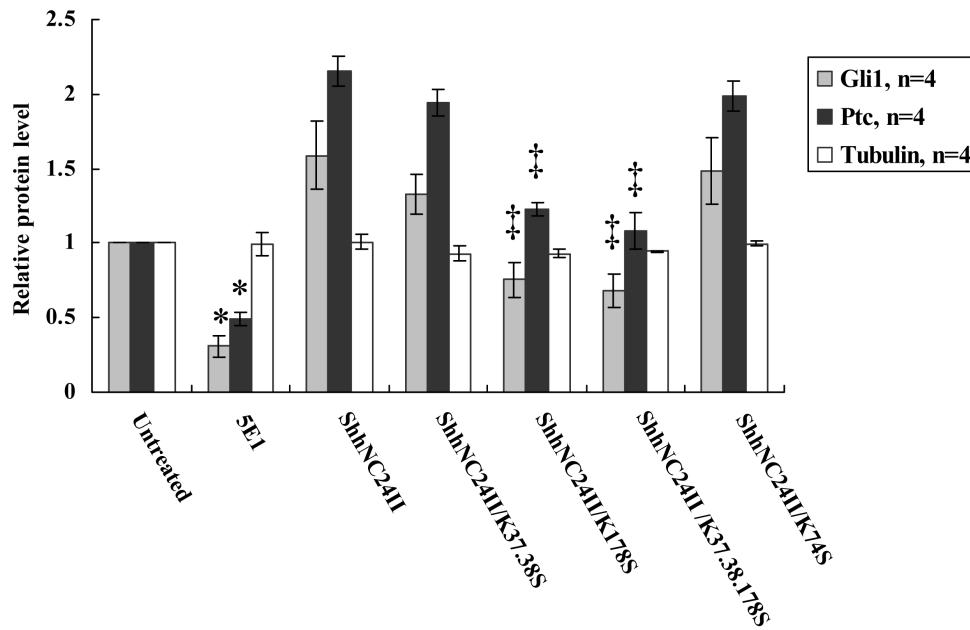


Figure 3-8. Biological functional assay of hShh proteins in PANC1 cells by Semi-quantitative RT-PCR and immunoblotting. To study the role of mutated hShhs in Shh signalling in a human model system, expression levels of Ptc and Gli1 were examined by both RT-PCR and Western blot in PANC1 cells after hShh proteins treatment. The PCR product agarose gel and immunoblotting data are provided in B and D respectively. Quantified results are given in C and E. Values are mean±S.D., n=9 in each group in RT-PCR experiments, * p -value <0.0001 compared to untreated group. ‡ p -value <0.001 compared to ShhNC24II group. In the Western blotting assay (n=4), * p -value <0.0001 compared to untreated group. ‡ p -value <0.001 compared to ShhNC24II group. The total extracted RNAs are shown in A.

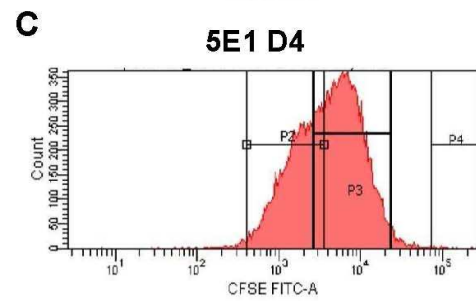
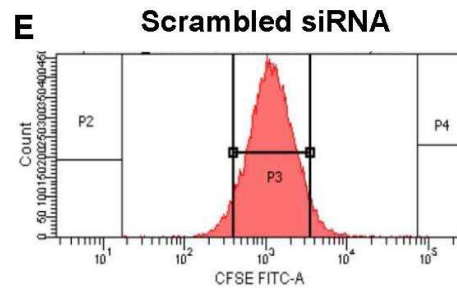
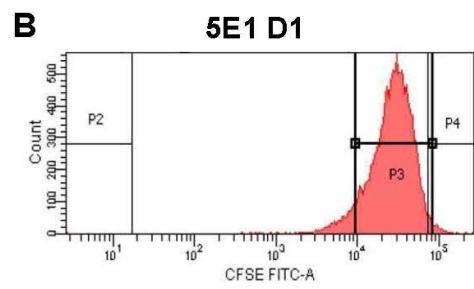
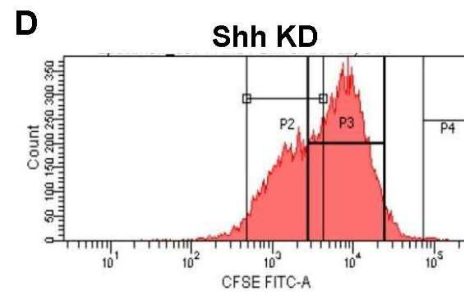
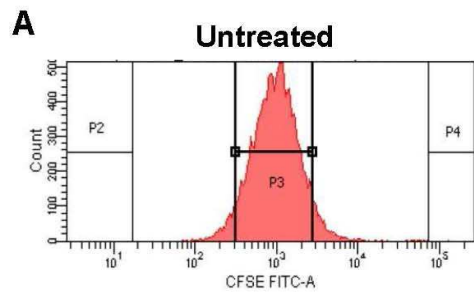
3-2-6. ShhNC24II or ShhNC24II/K74S but not ShhNC24II/K37.38.178S can rescue inhibition of PANC1 proliferation caused by hShh RNAi knockdown

To determine the effects on PANC1 proliferation of mutated hShhs that cannot interact with HSPGs properly, hShh RNAi KD and purified-Shh co-treatment were used, combined with cell proliferation analysis by CFSE incorporation followed by flow cytometry analysis. In this assay, cells are loaded at the start of the experiment with the dye CFSE which passively diffuses into cells; it is non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein (CF). During cell division, CF is distributed equally between the two daughter cells, which therefore have half the fluorescence of the parents. Dilution of CF by cell division over the time-course of the experiment is therefore a measure of proliferation. The histogram of flow cytometry is shown in Fig. 3-9 A-J. The quantitative data are provided in Fig. 3-9 K. I set out to investigate whether PANC1 proliferation is Shh-dependent by treating cells with 5E1, the inhibitory antibody of Shh, and the result was verified with Shh KD. According to my data from 30,000 cells in each condition, CF intensity in the 5E1 D1-treated group (5E1 treated on Day 1, Fig. 3-9 B) is ~30 times higher than untreated cells (Fig. 3-9 A), suggesting that untreated PANC1 cells reproduce 4-5 times more than 5E1-treated cells in 8 days. Moreover, the 5E1 D4 group (5E1 treated on Day 4, Fig. 3-9 C) used to mimic the kinetics of RNAi KD *in vivo*, shows a similar number as Shh KD with ~8 times higher CF intensity compared to untreated cells. Both results confirm that PANC1 proliferation is Shh-dependent. A scrambled siRNA (Fig. 3-9 E)

used as a negative control for Shh KD showed a similar profile to untreated cells.

Interestingly, 7 days after treatment the profile of flow cytometry in Shh KD cells treated with either active ShhNC24II or ShhNC24II/K74S revealed a similar pattern as untreated cells (MFI < 1000; Fig. 3-9 F, G), suggesting that both ShhNC24II and ShhNC24II/K74S proteins are capable to rescue reduced PANC1 proliferation caused by Shh KD ($P < 0.00001$).

On the contrary, purified ShhNC24II/K37.38S (Fig. 3-9 H) and ShhNC24II/K178S (Fig. 3-9 I) proteins added to culture medium showed less ability to rescue PANC1 proliferation caused by Shh KD with ~6 times increase in MFI (~4500) compare to untreated cells ($P < 0.00001$). Furthermore, ShhNC24II/K37.38.178S treatment of Shh KD cells showed a similar pattern with Shh KD alone (~8 times increase in MFI, ~6500) compared to untreated cells ($P < 0.00001$), indicating that ShhNC24II/K37.38.178S is defective in stimulating PANC1 proliferation compared to ShhNC24II and ShhNC24II/K74S. The above results indicate that HSPGs binding plays an important role in PANC1 proliferation through Shh signalling due to the fact that ShhNC24II or ShhNC24II/K74S can rescue inhibited PANC1 caused by hShh RNAi KD but the triple mutant ShhNC24II/K37.38.178S is unable to do so effectively.



← Proliferation

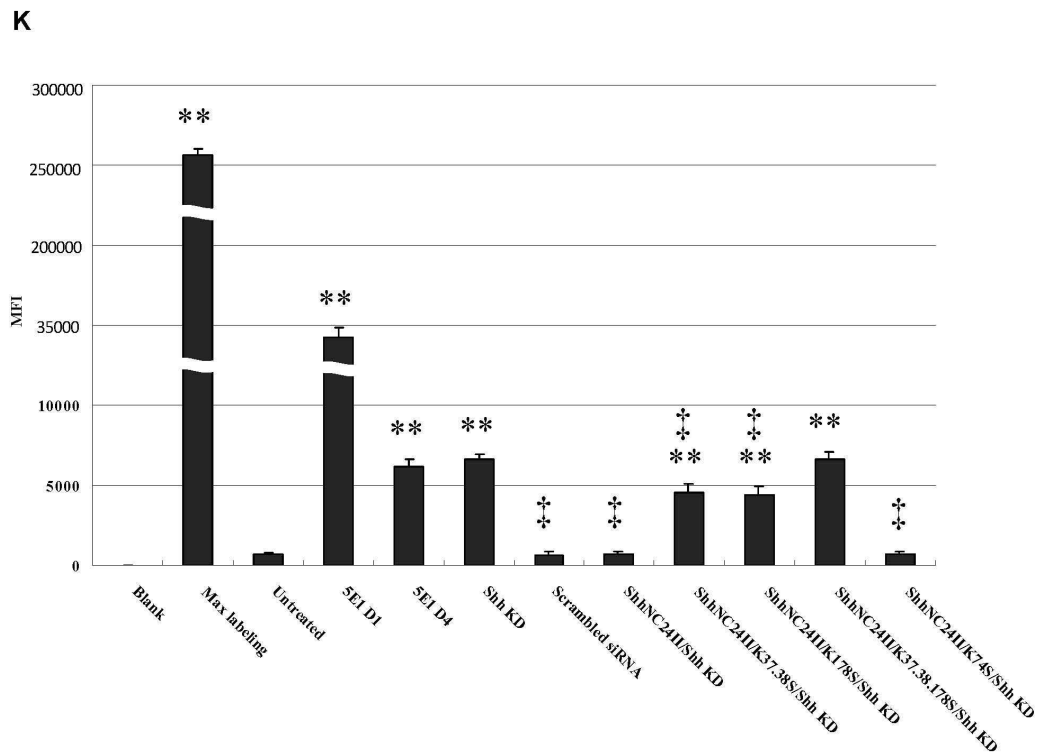
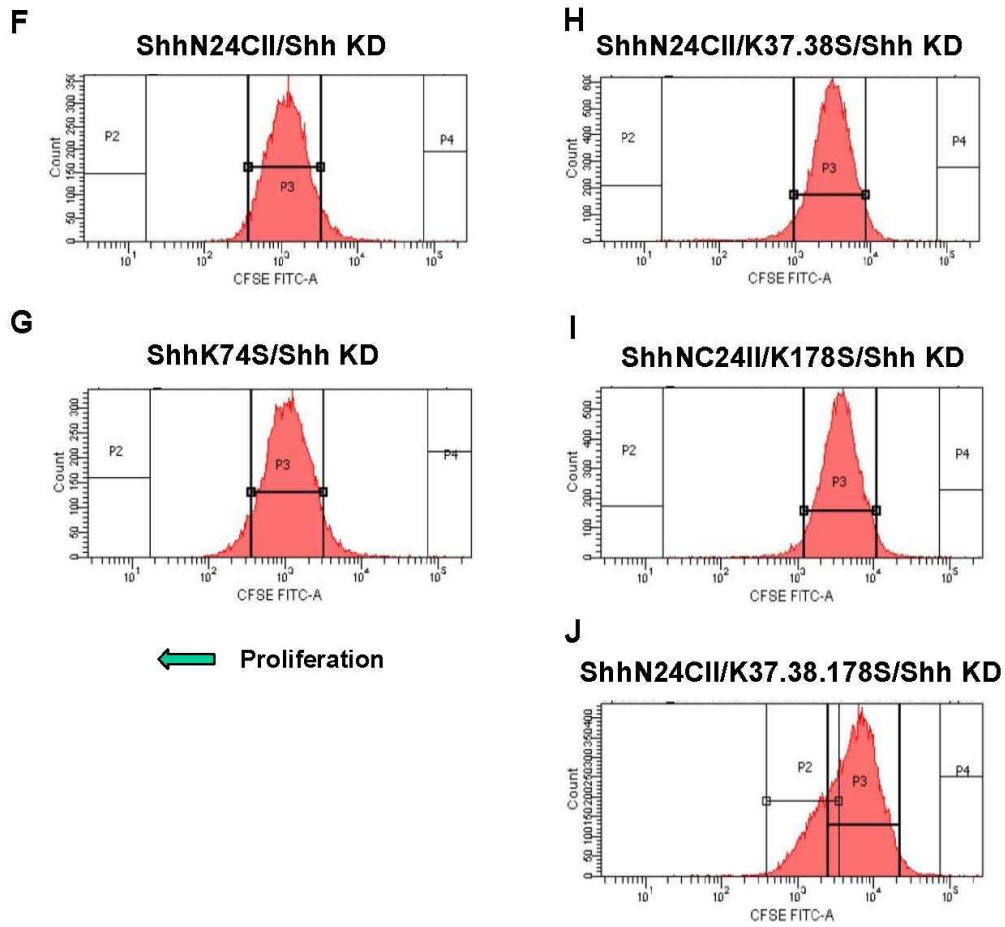


Figure 3-9. The role of mutated hShh proteins in PANC1 cell proliferation.

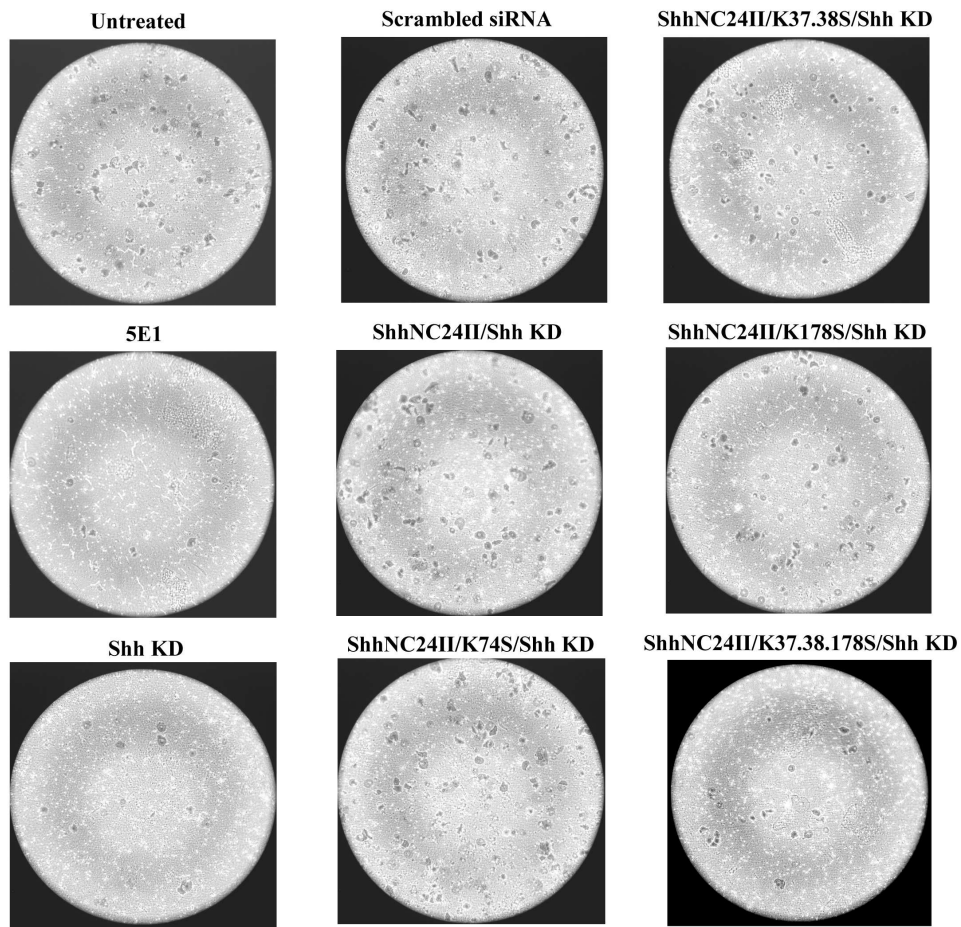
A-J show profiles from flow cytometry. The quantified data are given in K. Both 5E1 treatment on Day 1 (5E1 D1) and Shh KD were used to examine if PANC1 proliferation is Shh dependent. 5E1 treatment on Day 4 (5E1 D4) was used to mimic RNAi KD kinetics *in vivo*. The level of maximum labeling was determined by treating cells with CFSE just before flow cytometer assessment. To study the effect of different Shh proteins in PANC1 proliferation, 8 days after Shh siRNA transfection /hShh treatment the cells were analysed by flow cytometry. The X-axis of A-J and Y-axis of K show the level of CF intensity observed from 30,000 cells. The trend of cell proliferation is indicated by the green arrowhead. The experiments were repeated three times in triplicate, and statistical significance was measured by using the two-tailed t test (**, $P < 0.0001$ compared to untreated group; ‡, $P < 0.0001$ compared to Shh KD group).

3-2-7. ShhNC24II/K37.38.178S cannot rescue inhibited PANC1 invasion caused by hShh KD

To investigate the contribution of Shh mutations to invasive potential of PANC1 cells, I carried out invasion assays by exogenous Shhs treatment combined with endogenous Shh RNAi KD. To examine whether PANC1 invasion is dependent on Shh signalling I started by determining the effect on PANC1 invasion of 5E1 treatment. The results (Fig. 3-10) show a significant decrease in the number of invading cells after 5E1 treatment (33 ± 8 cells/well) compared

with untreated control cells (271 ± 25 cells/well). These data were then verified by Shh KD giving 43 ± 11 cells/well. These results suggest that the invasive ability of PANC1 cells is at least partly due to Shh signalling. Interestingly, when I combined Shh KD and either ShhNC24II or ShhNC24II/K74S in the invasion assay, a significant increase in the number of invasive PANC1 cells was observed (325 ± 32 and 302 ± 45 cells/well respectively) compared with Shh KD cells, suggesting that ShhNC24II or ShhNC24II/K74S are able to restore inhibited PANC1 invasion caused by Shh KD. In contrast, ShhNC24II/K37.38S and ShhNC24II/K178S combined with Shh KD showed significant decrease in the number of invasive PANC1 cells with 192 ± 15 and 191 ± 18 cells/well respectively. Furthermore, ShhNC24II/K37.38.178S combined with Shh KD reduced invasion to a level (86 ± 17 cells/well) similar to that observed for Shh KD cells, indicating that ShhNC24II/K37.38S, ShhNC24II/K178S and particularly ShhNC24II/K37.38.178S provide less stimulus for PANC1 invasion. To sum up, the results of the above studies indicate that HSPGs binding plays a crucial role in PANC1 invasion due to Shh signalling.

A



B

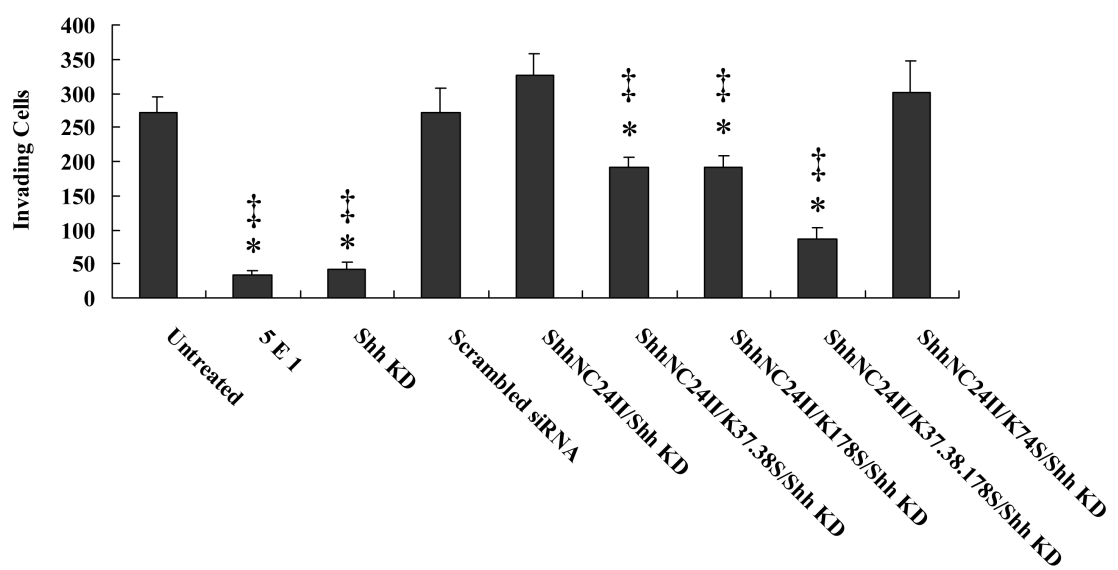


Figure 3-10. Biological function of mutated hShh proteins in PANC1 cells invasion. I set out to determine whether PANC1 invasion is Shh dependent using 5E1 treatment and Shh RNAi KD. To study the effect of different Shh proteins in PANC1 invasion, 72hr after RNAi transfection/Shhs treatment the cells were plated onto Matrigel invasion chambers for 24hr. Cells that had migrated from the upper to the lower side of the filter were photographed (A) and counted with a light microscope (10 fields/filter, B). The experiments were repeated four times, and statistical significance was measured by using the two-tailed t test (*, $P < 0.001$ compared to untreated group; ‡, $P < 0.0001$ compared to ShhNC24II/Shh KD group).

3-2-8. ShhC24II/K37.38.178S is defective in forming multimeric complexes in PANC1 cells

To evaluate the ability of mutated hShhs in formation of Shh multimeric complexes in extracellular matrix (ECM), gel filtration followed with Shh dot blot were carried out for PANC1 cells. The efficacy of the Shh KD was examined by Western blotting of culture medium (Fig. 3-11A). A 19kDa band was observed and more secreted Shh was found in medium from both untreated cells and scrambled RNAi-treated cells than from Shh KD cells, providing good evidence that Shh KD did reduce Shh expression. To prevent possible malfunction of purified Shh proteins on oligomerisation when applied exogenously to PANC1 cells, a mammalian overexpression vector-pcDNA-DEST40-hShh was used in the assay. By Shh dot blotting of gel

filtration fractions (Fig. 3-11B), the majority of secreted Shh in PANC1 control untreated culture medium forms large multimeric complexes and only a small portion of Shh migrates as monomers ($2.6 \pm 1.2\%$, $P < 0.0001$), confirming the results of a previous study [188]. On the contrary, in Shh RNAi-treated cultured medium the total amount of Shh was much reduced (>90% reduction compared with untreated control cells) and higher quantities of Shh monomers were detected ($12.7 \pm 4.5\%$, $P < 0.0001$), nearly 6 times higher than untreated control medium, whereas scrambled RNAi-treated medium shows a similar pattern to control medium with a ratio of $1.9 \pm 1.3\%$ monomer ($P < 0.0001$). As expected, ShhNC24II or ShhNC24II/K74S expression combined with Shh KD treatment, either had no difference from control or in most instances resulted in a slight decrease in Shh monomerisation with $1.2 \pm 0.9\%$ and $1.1 \pm 0.8\%$ respectively ($P < 0.0001$), providing evidence that transfected pcDNA-DEST40-hShh and pcDNA-DEST40-hShhK74S are capable to form multimeric complexes in PANC1 cells. Interestingly, when I analysed medium from pcDNA-DEST40-hShhK37.38S-expressing and pcDNA-DEST40-hShhK178S-expressing Shh KD cells, a strong increase in monomerisation was observed; $25.5 \pm 5.7\%$ and $20.4 \pm 4.6\%$ respectively ($P < 0.001$). Notably, a dramatic increase in monomer formation was detected in pcDNA-DEST40-hShhK37.38.178S-expressing Shh KD cells ($85.5 \pm 9.7\%$, $P < 0.001$). In combination, I conclude that wild type hShh and hShhK74S are able to form multimeric complexes when expressed in PANC1 cells, but hShhK37.38.178S is not, and the hShhK37.38S and hShhK178S mutants show an intermediate phenotype.

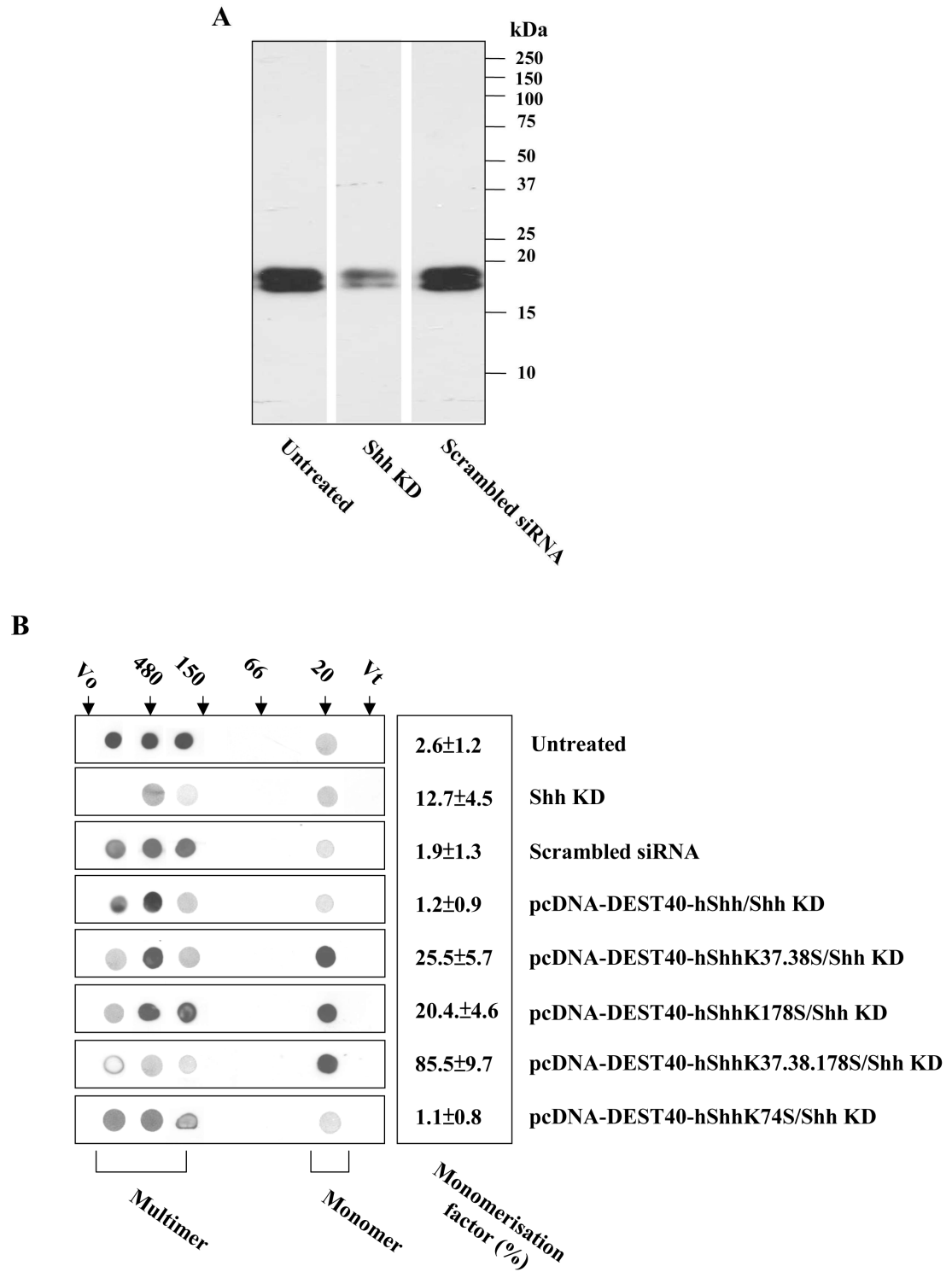


Figure 3-11. The role of mutated hShh proteins in Shh multimeric complex formation. 72hr after RNAi transfection, the culture media were TCA

precipitated and examined by Western blot for Shh (A). In B, to examine the role of mutated hShh proteins in Shh oligomerisation, 16hr after Shh siRNA treatment hShh overexpression vectors were transfected into the cells followed by 48hr incubation. Then, the media were subjected to gel filtration chromatography (Superdex200 10/300 GL column). After TCA precipitation, the fractions were probed by Shh dot blot (B). Monomerisation factor (%) = $(\text{Monomer} / \text{Monomer} + \text{Multimer}) \times 100\%$.

3-3. Discussion

HSPGs are tremendously diverse, due to the involvement of the genes encoding core proteins, the sulfotransferases, sugar transporters, glycosyltransferases, sulfatases and acetylating enzymes. As HSPGs interact with many growth factors and other molecules, analyses of mutants that interfere with individual steps of proteoglycan synthesis are difficult to interpret. To identify the functions of HSPGs for Shh signalling, I took an alternative approach by generating mutant Shh proteins that cannot bind heparin properly. In this work, I developed an *in vitro* system to express and purify hShh proteins. In both a bench-top elution method and heparin affinity column chromatography, I found that mutations in putative HSPG-binding sites of Shh, the Cardin-Weintraub motif (K37.38) and a novel residue K178, interfere with heparin binding significantly. The *in vitro* binding assay showed that heparin binding to wild-type Shh is disrupted by 0.54 M NaCl, but by only 0 - 0.06 M

NaCl for the K37.38.178S triple mutant. This is the first evidence indicating that not only the Cardin-Weintraub motif, but also K178 of Shh can be a significant residue involved in HSPGs binding.

C3H10T1/2 differentiation, carried out as assessed by alkaline phosphatase (AP) induction, is a standard assay for Shh activity. I found 2-4 times lower AP induction by mutants K37.38S (0.60 ± 0.14) and K178S (0.32 ± 0.09) compared to wild-type Shh (1.18 ± 0.11) and the control mutation K74S (1.19 ± 0.08), though high doses of Shh proteins were required, consistent with the lack of post-translational lipid modifications of the bacterially expressed proteins. These results indicate that AP induction by the Shh signalling pathway is associated with HSPGs and ligand interaction. Previous studies reported that Shh responses are mediated primarily by the transcription factors Gli1, Gli2, Gli3 [22-24,195] and the receptor Ptc. Unlike Gli2 and Gli3, which can function as either transcriptional activators or repressors, Gli1 only functions as an activator, and only one isoform of Gli1 has been identified. Here, I analysed the expression of Gli1 and Ptc in Shh-pretreated PANC1 cells. Consistent with the data above, both induced mRNA and protein levels of Gli1 and Ptc are lower following treatment by the mutant K37.38.178S than were acutely stimulated with wild-type Shh and K74S mutant. Ptc and Gli1 protein level are respectively 60% and 57% lower after treatment with the K37.38.178S triple mutant than for wild-type Shh. Together, these findings indicate that Shh-HSPGs interactions mediated by the Cardin-Weintraub motif and the novel residue 178K are significantly required for Shh signalling activity.

However, using a mammalian expression vector in both *C3H10T1/2* differentiation assay and PANC1 system would provide a better option to study the biological function of mutated Shh proteins; since this approach can preclude the possibility of misfolding of bacterially purified proteins.

A number of Shh responsive genes have been identified, including genes that are involved in proliferation and tissue patterning [196-202] e.g. *Gli2*, *N-myc*, *Gli3*, *Cyclin D1*, *Cyclin D2* and *Bmi-1*. In addition, it has also been shown that *Cyclin D1*, *Cyclin D2* and *Bmi-1* gene targets that require Shh-HSPG interactions are significant in tumour stem cell biology [196,197]. Also, it is interesting to note that Shh-dependent cancer growth can be stimulated via glypican overexpression in several cases, e.g. prostate cancer, pancreatic carcinoma and rhabdomyosarcoma [203,204]. In this work, I asked whether interactions with HSPGs can modulate the pattern of Shh-responsive gene induction and the biological responses to Shh.

It has been reported that 5E1 is a specific inhibitor of the Shh pathway by binding to Shh as a neutralising antibody and inactivating Shh signalling [205]. I employed 5E1 to block the Shh signalling pathway in PANC1 cells and to determine the effect of the pathway on proliferation and invasiveness of PANC1 cells. A significant decrease in tumour cell proliferation (4-5 times lower within 8-days) and an eightfold reduction in invasiveness were observed, suggesting that Shh signalling has a critical and central role in pancreatic tumour cell growth and contributes to malignant transformation.

By flow cytometry, I directly demonstrated that changing wild-type Shh to either K37.38S or K178S mutant results in a reduced proliferation pattern; an even more marked effect was noted with the K37.38.178S triple mutant, which revealed a threefold reduction in tumour proliferation within 8 days. These findings indicate a close relationship between the Shh-HSPGs binding and the proliferation ability of pancreatic cancer cells.

In the invasion assay, I observed a decrease in invasive cells by K37.38S (192 ± 15 cells/well) or K178S (191 ± 18 cells/well) treatment compared to wild-type Shh (325 ± 32 cells/well) and control K74S mutant (302 ± 45 cells/well). Not surprisingly, the triple mutant K37.38.178S resulted in a fourfold reduction in invasive cells compare with wild-type Shh and control mutant. These data indicate that Shh interacts with HSPGs in PANC1 cells to influence the invasive ability of the pancreatic cancer cells.

Together, these findings suggest that Shh-HSPGs interactions promote PANC1 cells oncogenic properties at multiple levels, including both proliferation and invasion, highlighting the widespread significance of proteoglycans in tumorigenesis and also pointing out an important mediator of the malignant biological behaviour of pancreatic adenocarcinomas in humans. However, to prevent from possible malfunction of bacterially purified proteins (e.g. misfolding of purified proteins) using a mammalian expression vector would be a better way to examine the functional role of mutated Shh proteins in

PANC1 tumourgenesis and malignant transformation.

There are two potential mechanisms to explain the effects of proteoglycans on Shh-induced proliferation and invasion in PANC1 cells. One is that HSPGs provide Shh accurate trafficking and targeting on receiving cells. Previous studies using mutations in the glypican Dally-like, mutations in the *Ext* gene family (the HSPG-synthesising enzymes) and truncation of the Cardin-Weintraub motif have suggested that HSPGs are critical for appropriate localisation of Hh proteins [55,138,139,206]. A second potential mechanism is that binding of Shh to HSPGs modulates stability of Shh in ECM via forming a multimeric complex. In this work, to address this possibility I asked whether mutated Shh proteins had altered ability to form multimers.

In the oligomerisation study, by expressing the Cardin-Weintraub K37.38S mutant instead of wild-type Shh or control mutant K74S I observed an increase in Shh monomer ratio in the medium. Interestingly, the K178S mutant resulted in a similar pattern as the Cardin-Weintraub mutant. From the above data, here I hypothesise for the first time that K178 of Shh may play key a role in Shh multimeric complex formation via binding with HSPGs. Consistent with this, in the 3D structure of Shh, K178 is located close to the HSPG-binding groove containing the Cardin-Weintraub motif (K. Grobe, personal communication). The further increased monomer ratio (over 80%) found for the K37.38.178S mutant is in agreement with the suppressed initiation and maintenance of PANC1 cells revealed by the above proliferation and invasion data.

Thus, the above data provide supportive evidence leading to the idea that one mechanism by which Shh-HSPGs interactions stimulate Shh signalling is by forming stabilised multimeric complexes in ECM [188]. On the other hand, a previous study in transgenic mice has shown that mutations in the Cardin-Weintraub motif of Hh proteins interfere with proteoglycan binding, but do not alter Ptc affinity [207]. However, in this case we cannot exclude the possibility that reduced PANC1 proliferation and invasion induced by the K178 mutant is also due to the loss of Ptc interaction. In summary, the findings presented in this work provide a new perspective that Shh-HSPGs interactions promote proliferation and invasion in PANC1 cells, via forming a multimeric complex in ECM to facilitate stability and solubility of Shh proteins.

CHAPTER 4

4. The Role of Hhat in Human Shh Signalling

4-1. Introduction

Post-translational lipid modifications are known in other secreted signalling molecules, i.e. the Wnt protein family that is another conserved family in vertebrates and invertebrates, as well as in the Epidermal Growth Factor Receptor (EGFR) ligand Spitz [208]. However, Hh proteins are unique in terms of being dually lipid-modified with a fatty acid and cholesterol to be fully active [159].

Palmitoylation is the attachment of fatty acids from Palmitoyl-Coenzyme A (Pal-CoA) as a lipid donor, usually to cysteine residues of proteins. Unlike myristoylation and farnesylation, palmitoylation provides modified cytoplasmic proteins accurate trafficking from internal membranes to the plasma membrane [6] and controls their targeting to membranes or membrane subdomains, affects protein–protein interactions, or influences the stability of proteins. In addition, studies demonstrate that palmitoylation can facilitate the efficiency and specificity of signalling through not only correctly guiding a signalling molecule to its target within the cell but also membrane-anchoring at specific cell surface microdomains/lipid rafts [59,146]. Moreover, it has been shown that dual lipidation is critical not only for the interactions between Hh and Ptc but also for forming a suitable Hedgehog (Hh)- heparan sulfate proteoglycans (HSPGs) complex to target at the Hh-receiving cell [146]. It is

now appreciated that Ptc might be located in lipid rafts/microdomains which provide platforms for signal transduction and intracellular sorting [160]. Hence, the interactions between particular HSPGs, Hh and Ptc are significant to Hh spreading through the epithelium surface, as well as precise Hh reception.

It is evident that the N-terminal palmitoylation of Hh is facilitated by the product (Hhat) of the *hedgehog acyltransferase* gene (also known as *skinny hedgehog*, *sightless*, *central missing* and *raspberry*) [141]. However, the mechanism of this N-acylation is still unclear [209]. Structurally, Hhat shares homology with the membrane-bound O-acyltransferases (MBOAT) family (Fig. 4-1), the members of which are enzymes that generally catalyse the addition of a fatty acid to a hydroxyl group [29]. Hhat also shares homology with Porcupine (Porc) in *Drosophila* and its *C. elegans* homologue Mom-1 [29], two putative acyltransferases that are also part of the MBOAT protein family and are responsible for the palmitoylation of Wg, a morphogen involved in embryonic patterning in *Drosophila*, and its human homologues Wnts [29,59]. Another MBOAT family member, GOAT, has been implicated in metabolic activation of the peptide hormone ghrelin by specifically octanoylating ghrelin on its critical serine-3 residue [61].

From various studies we hypothesise that the mechanism by which Hhat adds a palmitate to the N-terminal of the cysteine-24 residue in Shh [34] may be solely enzymatic, chaperone-like or a combination of both. For instance, Hhat contains the characteristic histidine residue conserved in almost all members

of the MBOAT family [29]. It is believed that this conserved histidine residue-H379 may represent the active site of the putative acyltransferase [210]. Based on a very recent study, the Hhat H379A mutant retains 50% activity compared with wild-type Hhat, suggesting that this conserved Histidine is not the only factor required for catalysis. Combined with the fact from kinetic analyses that purified H379A Hhat showed similar affinity to Pal-CoA as wild-type Hhat, suggesting H379 may involved in Shh recognition and interaction. Moreover, point mutation of the adjacent residue- W378A caused a more reduced Hhat activity. Also, the fact that Hhat being a member of palmitoyl acyltransferases (PATs) with a known enzymatic function in palmitoylating a group of proteins [14] leads the argument towards the mechanism being enzymatic. On the other hand, Pal-CoA, the co-substrate of acyltransferases, instead of being free in the cell is bound to the acyl-CoA binding protein (ACBP) and therefore may require a transporter that facilitates its entry into the lumen of the secretory pathway where palmitoylation of Shh presumably occurs [162]. Also, the fact that Hhat is a multi-spanning transmembrane protein raises the speculation that it may play a role as a transporter of Pal-CoA.

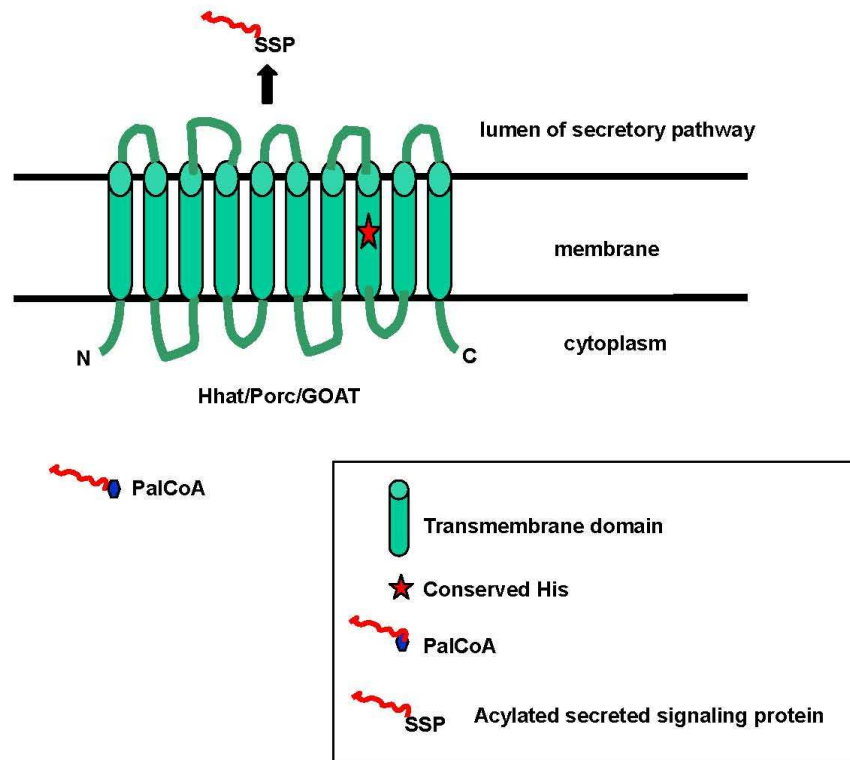


Figure 4-1. MBOAT family members topology. MBOAT (membrane-bound O-acyltransferase) family members that promote protein acylation - Hhat, Porc and GOAT - contain multiple membrane-spanning domains. These palmitoylacyltransferases (PATs) are responsible for attaching fatty acids (originating in the cytoplasm in the form of activated CoA esters) to secreted signalling proteins (SSP), such as Hhs, Wg/Wnts and ghrelin, within the lumen of the secretory pathway. The histidine shown is conserved in almost all members of the MBOAT family and may contribute to the active site of the putative acyltransferase (from ref.147).

4-2. Results

4-2-1. Human Hhat antibody generation

To determine the localisation of endogenous human (h) Hhat and to study the biological role of hHhat *in vivo*, we set out to generate an hHhat antibody based on an anti-peptide approach. In addition, to enrich the diversity of antibodies that are produced and to short-circuit potential failure if a single antigen does not produce an immune response, a multiple-peptide coimmunisation approach was used. Other benefits from the multiple-peptide immunisation strategy include: 1) Higher chance of finding best antibodies from the pool of antibodies generated. After isolation, the individual peptide antibodies can be characterised for application such as immunoprecipitation. 2) Lower cost than separate protocols. 3) Increase the chance for the ability to generate more than one peptide antibodies to one protein due to exposure of greater number of selected target epitopes which can be isolated using specific peptide affinity purification.

In this study, two predicted extramembrane domains from hHhat were chosen as suitable epitopes (shown in Fig. 4-2, underlined with blue line). The sequences of the peptides are: KQMQQQEHD^{SLKASL} (a.a.227 – a.a.241) and CYSHVGI^{AWAGTYATD} (a.a.478 – a.a.493). These were coupled to KLH carrier and co-immunised into rabbits (Materials and Methods). After characterising potential antibodies by examining subcellular fractionation of the antigens (Section 4-2-2), immunoblotting assays were performed to determine

the specificity of the antibody.

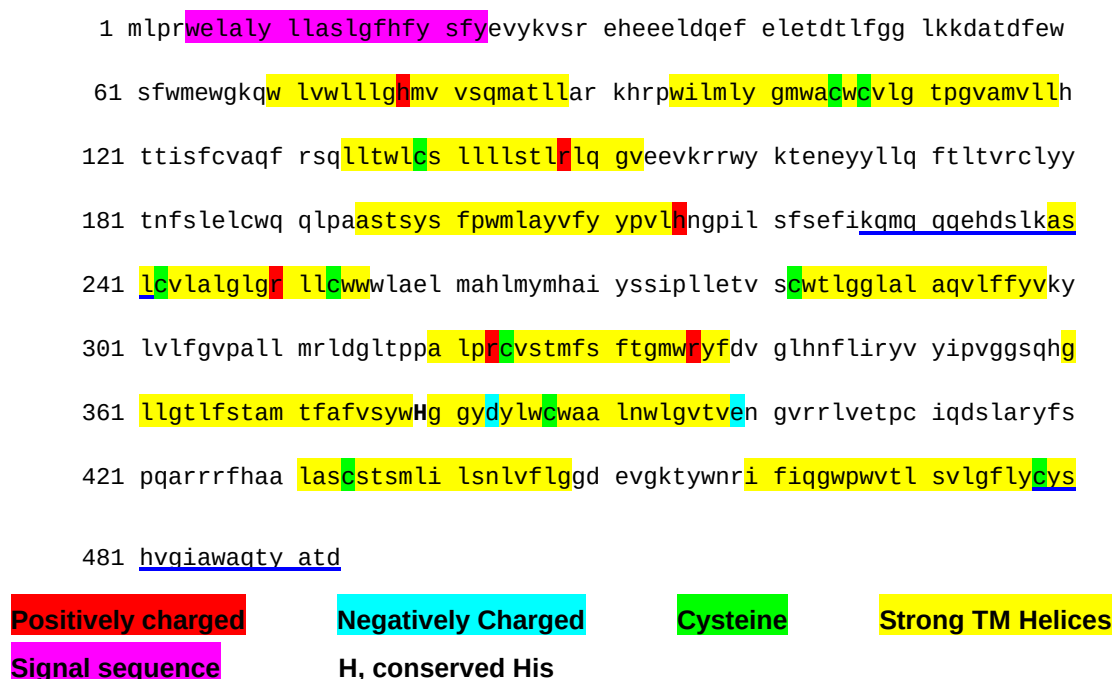


Figure 4-2. Suggested model for the transmembrane domains of hHhat.

The sequences of two peptides used to generate hHhat antibody are underlined with blue lines. The potential transmembrane (TM) domains of hHhat are highlighted in yellow (C. Palmer, personal communication). Positively and negatively charged residues in the transmembrane domains are highlighted in red and light blue respectively. The histidine 379 residue conserved in almost all members of the MBOAT family (shown as **H**) may contribute to the active site of the putative acyltransferase.

4-2-2. Characterisation of hHhat antibody

To separate cytosolic and membrane-associated proteins, HEK293a cells were subjected to S100/P100 fractionation and immunoblotting. An hHhat-EGFP mammalian expression construct was prepared; the clone map is shown in Fig. 4-3. To characterise the hHhat antibody, un-transfected, hHhat-EGFP- or mGup1-EGFP-transfected cells were subjected to subcellular fractionation followed by Western blotting (Fig. 4-4). Fig. 4-4A shows that a single protein (~32 kDa) was recognised by our hHhat antibody in the P100 but not the S100 fraction, showing that this endogenous protein is membrane-associated. This molecular weight corresponds to that reported previously [211]. This is consistent with a multi-spanning protein with predicted size of 57 kDa showing increased mobility in SDS/PAGE due to elevated SDS binding. To examine the specificity of this antibody, we used hHhat-EGFP- or mGup1-EGFP-transfected HEK293a cells, and analysed them by immunoblotting for GFP and Hhat. Anti-GFP immunoblotting shows expression of mGup1-EGFP and hHhat-EGFP, representing the transfection efficacy (Fig. 4-4B and C, left panel). Immunoblotting with anti-hHhat showed that this antibody recognised both endogenous hHhat and transfected hHhat-EGFP fusion protein (~58 kDa in Fig. 4-4B, right panel) but not exogenous mGup1-EGFP fusion. These results demonstrate the specificity of this antibody for hHhat without cross-reaction with mGup-1, another member of the MBOAT family which shares high homology to Hhat.

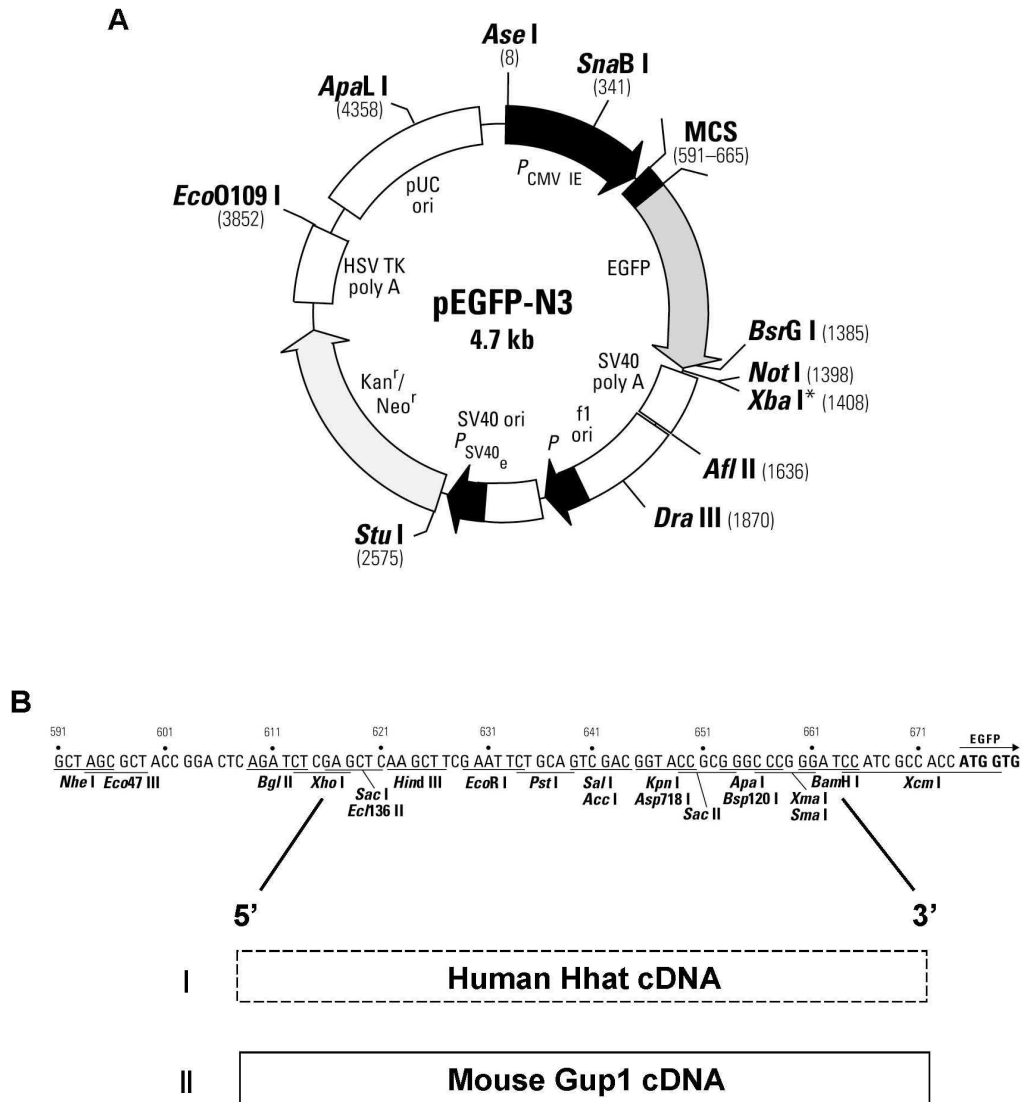


Figure 4-3. Restriction Map (A) and Multiple Cloning Site (MCS, B) of pEGFP-N3 (Unique restriction sites are in bold). The Not I site follows the EGFP stop codon. The Xba I site (*) is methylated in the DNA provided by BD Biosciences Clontech. Human Hhat cDNA and mouse Gup1 cDNA were inserted between XhoI and BamHI sites based on a PCR method as shown in I and II respectively.

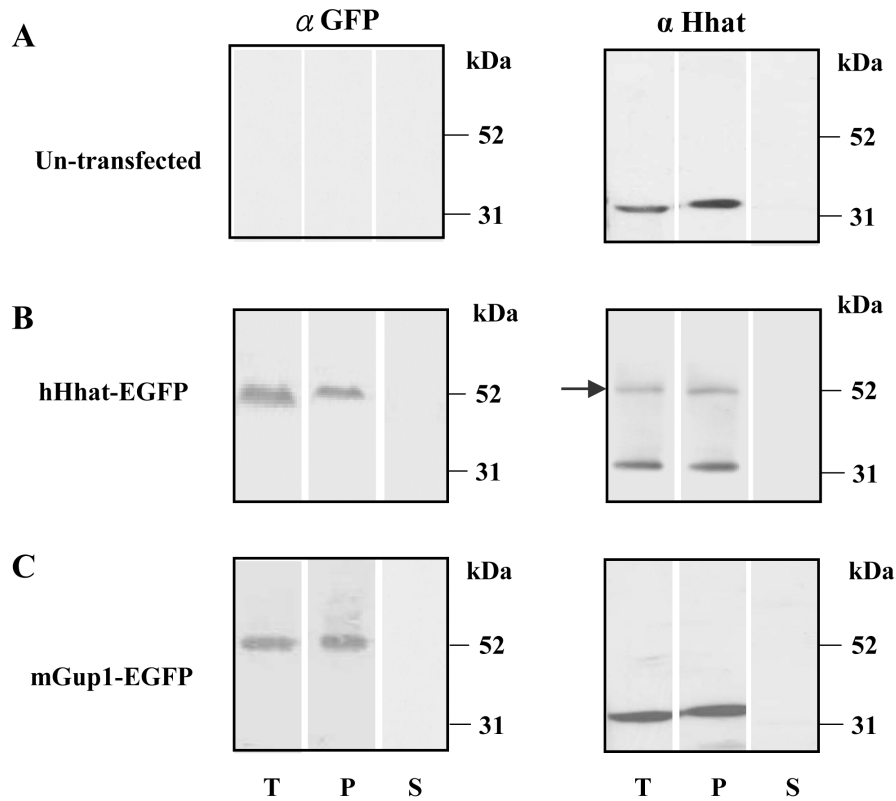


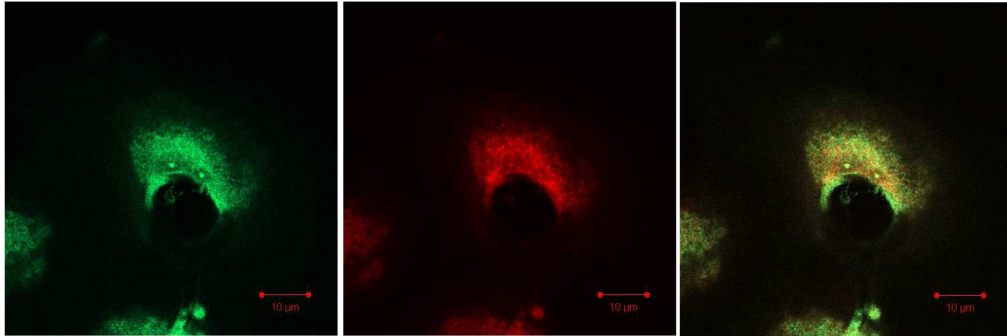
Figure 4-4. Characterisation of hHhat antibody. A. S100/P100 separation in un-transfected HEK293a cells, showing that a single ~32 kDa protein was shown in total cell lysates is a membrane protein but not a cytosolic protein. T, total cell lysate; P, P100 membrane fraction and S, S100 cytosolic fraction. B and C show the specificity of our Hhat antibody; hHhat-EGFP- or mGup1-EGFP-transfected HEK293a cells were used. The left panel is a Western blot for GFP showing transfection efficacy, whereas the right panel shows a Western blot with anti-hHhat demonstrating that our hHhat antibody recognises both endogenous hHhat (~32 kDa) and hHhat-EGFP fusion protein (~58 kDa, shown by arrow) located in the membrane fraction, but not mGup1-EGFP fusion, a mouse homologue of Hhat.

4-2-3. Intracellular localisation of endogenous hHhat to ER

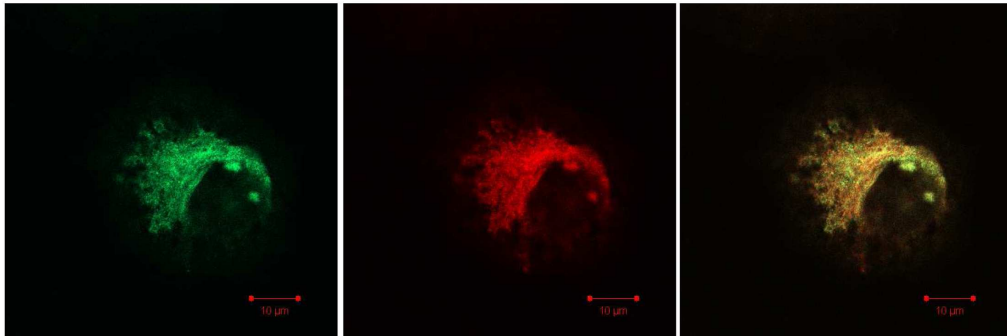
To determine the intracellular localisation of endogenous hHhat in PANC1 cells, the hHhat antibody, anti-GM130 and anti-PDI were used for immunofluorescence confocal microscopy. Here, different patterns of Hhat staining in Fig. 4-5A and C (also Fig. 4-5B and D) were observed is the consequence of distribution of ER and Golgi complex appeared *in vivo* differently. Confocal sectioning of stained PANC1 cells showed that the hHhat protein was primarily in the ER (Fig. 4-5A). Z-sections taken every 0.5 μm through the cell showed little, if any, hHhat within the Golgi complex (Fig. 4-5C). These results were verified using hHhat-EGFP-transfected PANC1 cells; a similar distribution was observed as for endogenous hHhat, confirming that hHhat localised mainly in the ER but not in Golgi apparatus (Fig. 4-5B and D). The Pearson product-moment correlation coefficients (kindly provided by Dr. Marek Cebecauer) are shown in Fig. 4-6 (**, $P < 0.001$).

In statistics, the Pearson product-moment correlation coefficient (referred to as the PMCC, and typically denoted by r) is a measure of the correlation (linear dependence) between two random variables X and Y , giving a value between +1 (highly correlated) and -1 (completely uncorrelated). As expected, PMCC yielded significant linear dependence between endogenous Hhat and ER as well as between extrogenous Hhat-GFP and ER, but neither between endogenous Hhat and Golgi complex nor between extrogenous Hhat-GFP and Golgi complex.

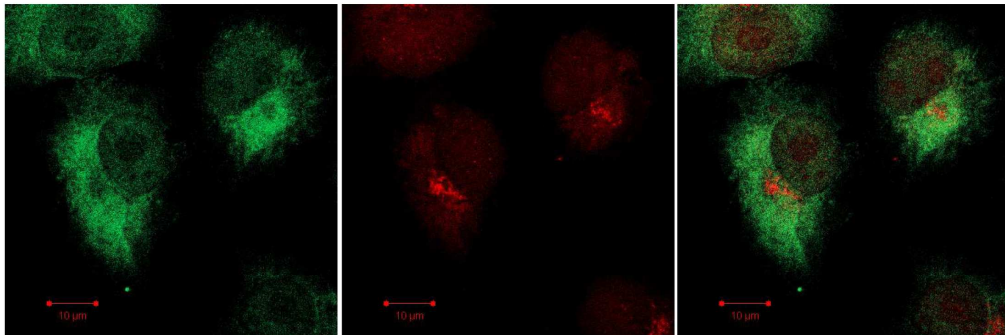
A **endo Hhat/PDI**



B **Hhat-GFP/PDI**



C **endo Hhat/GM130**



D **Hhat-GFP/GM130**

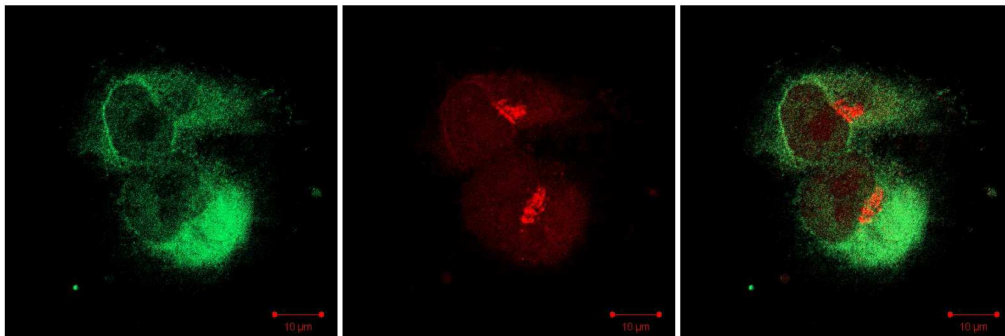


Figure 4-5. Localisation of hHhat in PANC1 cells. Localisation of hHhat in PANC1 cells was indicated by confocal microscopy combined with immunofluorescence of endogenous hHhat (green in A, C), ER (red in A, B) and Golgi (red in C, D) markers; and the result was verified in hHhat-EGFP-transfected cells (green in B, D). Taking A/B and C/D together shows that endogenous hHhat and hHhat-EGFP show a similar distribution pattern in the cells, localising in ER mainly but not in Golgi apparatus.

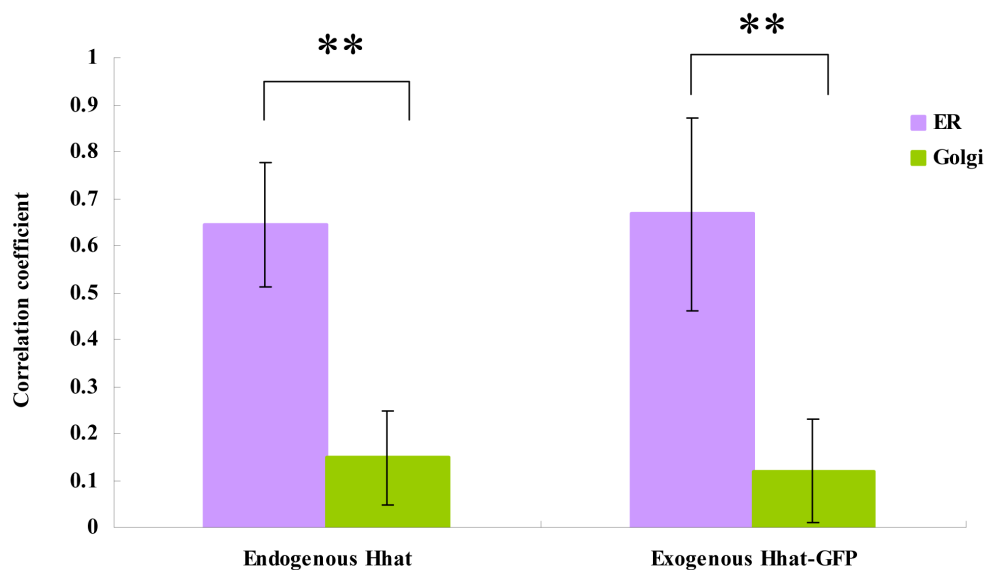


Figure 4-6. Pearson product-moment correlation coefficients (PMCC) for hHhat confocal microscopy. PMCC estimates the strength of the linear relationship between two random variables. The results here show 0.65 ± 0.13 in endoHhat/PDI, 0.67 ± 0.20 in Hhat-GFP/PDI, 0.15 ± 0.10 in endoHhat /GM130 and 0.12 ± 0.11 in Hhat-GFP /GM130; confirm and

extend previously published data reporting the localisation of transfected Hhat-HA in COS-1 cells [12].

4-2-4. Knocking down hHhat in PANC1 cells causes reduction in Shh palmitoylation and cellular retention

To determine whether Hhat is essential for Shh palmitoylation, I established conditions for effective knockdown (KD) of hHhat in PANC1 cells and combined this with an *in vitro* Shh palmitoylation assay. The siRNA KD of hHhat was examined by immunoblotting of hHhat; α -tubulin was used as a loading control. In addition, to determine the specificity of hHhat siRNA two negative controls were used: a scrambled siRNA (Hhat-Scr) and a specific control mutated hHhat siRNA (Hhat-Mut) which contains mutations from the original hHhat siRNA in the 10th, 13th and 19th nucleotide to preclude siRNA activity. My results show that optimised hHhat RNAi KD results in a >70% decrease in hHhat protein expression in PANC1 cells (Fig. 4-7A and 4-7B), whereas with hHhat-Mut siRNA the hHhat protein level was unaffected, and similar results were observed with Hhat-Scr RNAi treatment (Fig. 4-7A and 4-7B).

Next, I established a cell-based Shh palmitoylation assay based on the Cu(II)-catalysed azido-alkyne cycloaddition (CuAAC) reaction, also called click-chemistry (The relevant reagents were kindly provided by Dr. Tate EW

and Jovanovic B) [181]. The traditional approach for tracking protein lipid-modification is based on radioisotopic labelling which has often been considered time-consuming, usually taking days, weeks or even months to process. As an alternative approach, click-chemistry was used here. Click-chemistry was defined as bond-forming reactions useful for rapid assembly of molecules with desired function [242]. Copper and ruthenium are the catalysts commonly used in the reaction. Copper catalysts (Fig. 2-1) accelerate the reaction of azides with terminal alkynes by $\sim 10^7$ relative to the uncatalysed version, making it conveniently fast at room temperature [234]. These transformations are easy to perform, give rise to their intended products in very high yields with little or no byproducts, and is insensitive to oxygen and water. In fact, in several instances water is the ideal reaction solvent, providing the best yields and highest rates [233].

In short, in this study proteins metabolically incorporate the palmitate analogue heptadec-16-ynoic acid (YnC15) and are detected following reaction with an azido-TAMRA-biotin tag. Palmitoylation can be monitored by fluorescence imaging of the tag and also by anti-Shh immunoblotting which detects a slightly larger Shh band due to tag addition. The fluorescence result (Fig. 4-7C) shows a single ~ 22 kDa fluorescent protein, YnC15-Shh-N, immunoprecipitated by anti-Shh 5E1 from both cell lysates and medium. In addition, this fluorescent protein was more abundant after hHhat-Mut RNAi treatment compared to hHhat KD treatment, indicating that knocking down hHhat inhibits palmitate analogue YnC15 incorporation into endogenous Shh. Moreover, the same

samples were examined by immunoblotting for Shh; in both culture medium and cell lysates, YnC15-Shh was detected as a ~22 kDa protein (shown by arrow in Fig. 4-7C). In the hHhat KD cell lysate, there was less YnC15-Shh compared to the hHhat-Mut sample, corroborating the result with fluorescence imaging. On the other hand, in the medium from hHhat KD cells there was more Shh (~19 kDa) compared with cells treated with hHhat-Mut RNAi (Fig. 4-8E), suggesting that knocking down hHhat enhances Shh release into the medium, albeit presumably in unacylated form. Combined with the fact that less endogenous Shh was observed in lysates from hHhat KD cells compared to hHhat-Mut-treated cells, this supports the interpretation that knocking down hHhat suppresses Shh retention.

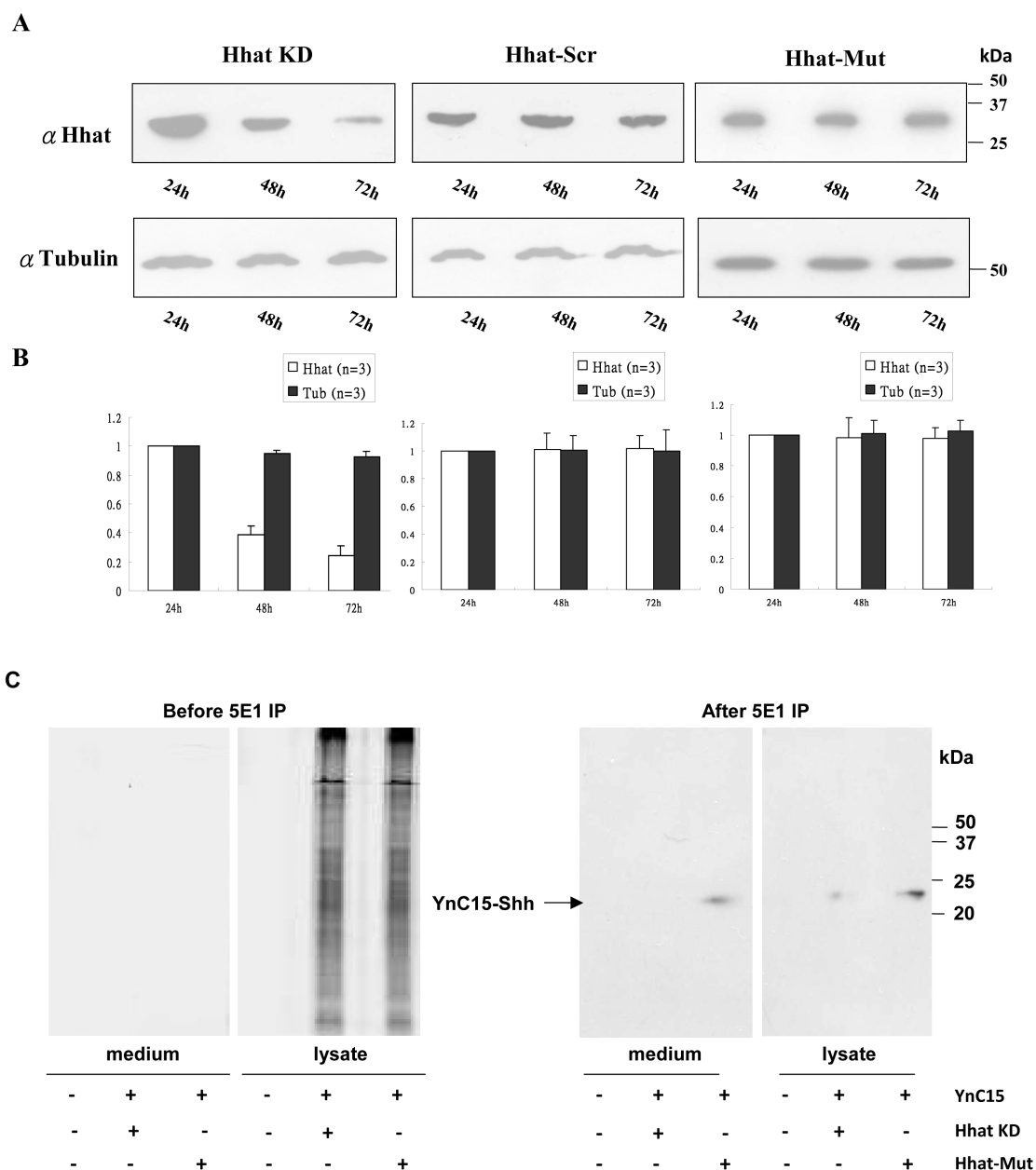


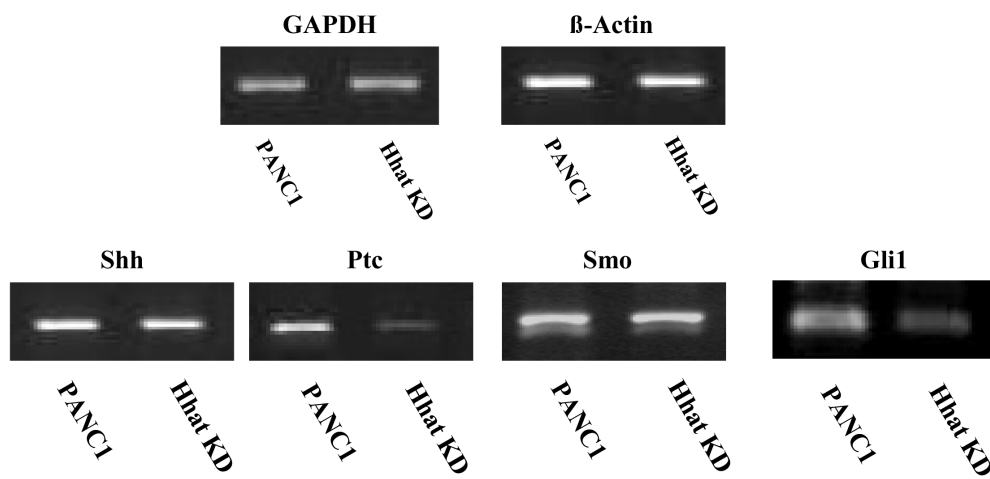
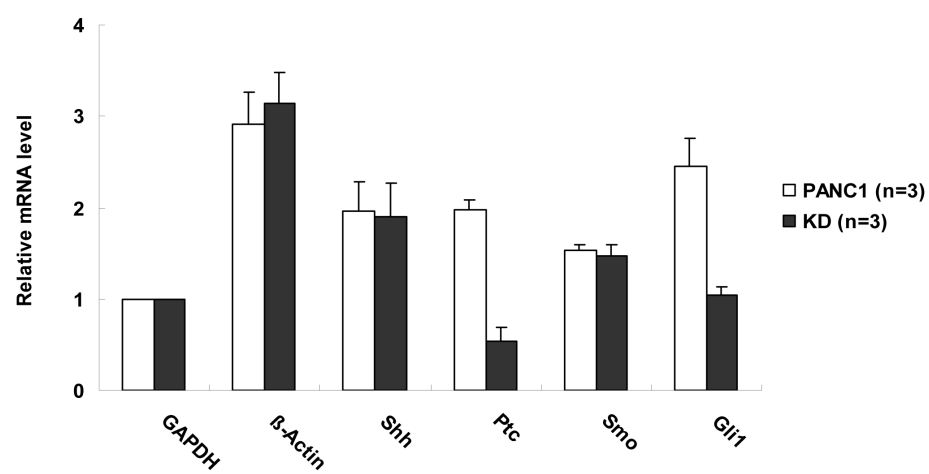
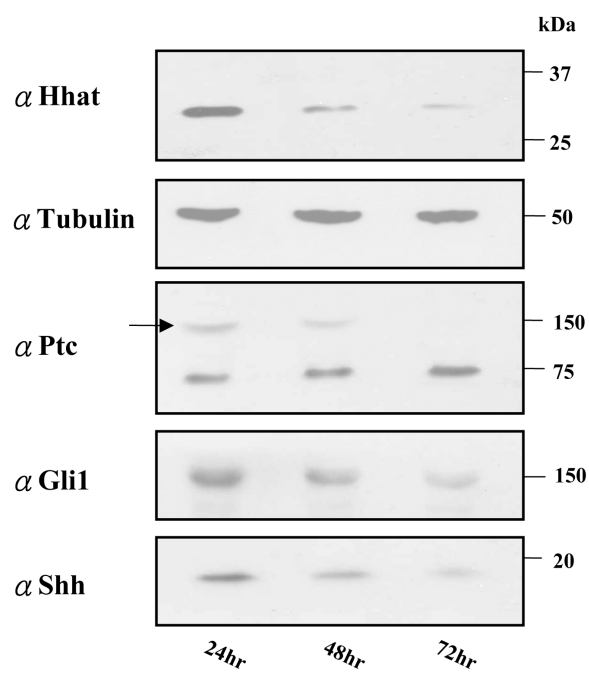
Figure 4-7. hHhat RNAi knockdown in PANC1 cells. To examine hHhat RNAi KD efficacy in PANC1 cells, a Western blot for Hhat was processed after 24hr, 48hr and 72hr siRNA transfection. Tubulin was used a loading control (lower panel in A). Quantitative results are shown in B. To confirm the role of hHhat in Shh palmitoylation, click-chemistry was carried out to detect palmitoylation *in vivo* (C). After 5E1 immunoprecipitation (IP, right), YnC15 (a

palmitate analogue)-labeled Shh was detected as a single band by fluorescence (arrow). Compared to Hhat-Mut-treated cells, YnC15-Shh was reduced in hHhat KD condition in both medium and cell lysates, suggesting that hHhat KD decreases Shh palmitoylation in this *in vitro* assay.

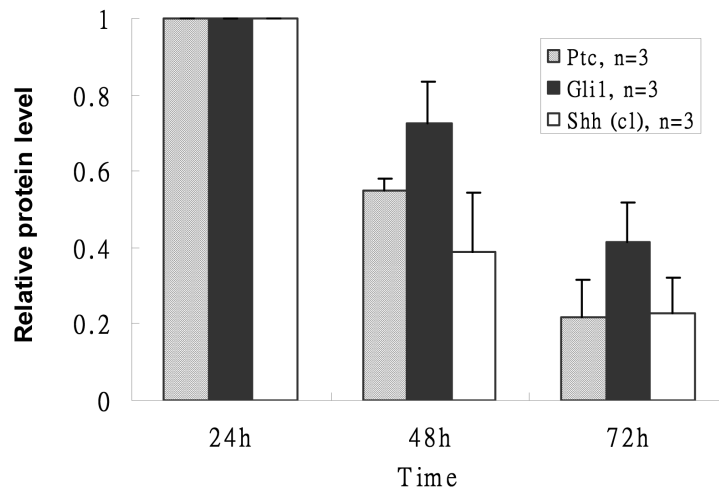
4-2-5. Knocking down hHhat causes decreased signalling through the Shh pathway in PANC1 cells

To determine whether hHhat (and hence palmitoylation of Shh) is required for Shh signalling activity, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting were performed on a hHhat KD time-course experiment; GAPDH and β -Actin were used as loading controls. In hHhat KD conditions, both Patched (Ptc) and Gli1 were found to be decreased in mRNA level by >70% and >50% respectively, whereas Shh and Smoothed (Smo) showed no significant difference (Fig. 4-8A and B). Immunoblotting was carried out to verify these results at the protein level, showing that hHhat decreased up to 70% in protein level after hHhat siRNA treatment, while Ptc and Gli1 were reduced by ~70% and ~50% respectively (Fig. 4-8C and D), consistent with RT-PCR. All these data suggest that knocking down hHhat causes decreased signalling through the Shh pathway in PANC1 cells. Interestingly, when I examined Shh by immunoblotting of cell lysates, cell-associated Shh was reduced ~70% in hHhat KD cells (Fig. 4-8E). This reduction is not due to alteration in Shh mRNA level (Fig. 4-8A) but by

reduced protein association with the cell membrane, supporting the previous suggestion that un-palmitoylated Shh has reduced ability in cell membrane association.

A**B****C**

D



E

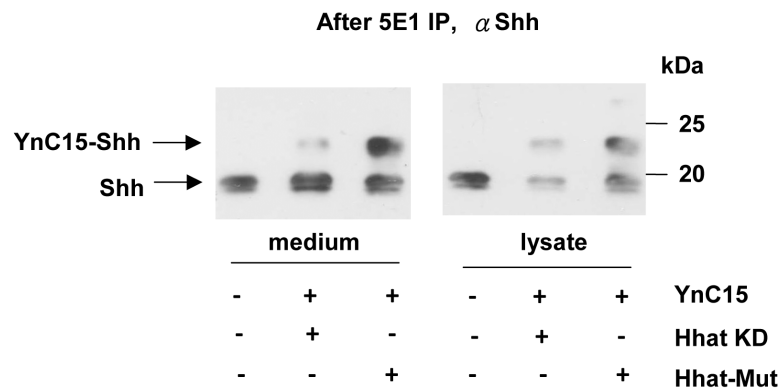


Figure 4-8. Effect of hHhat on Shh modification and signalling in PANC1 cells. To study the role of hHhat in Shh signalling, expression level of Ptc and Gli1 were examined by both RT-PCR and Western blot after hHhat KD (A, C). Quantification is provided in B and D. Interestingly, cell-associated Shh is reduced after hHhat KD (C, bottom panel) but Shh mRNA level remains unchanged (A), suggesting that hHhat KD reduces Shh retention to cell membranes. In E, 48hr after RNAi KD samples were labeled with YnC15 (a palmitate analogue) for 48hr and processed by click-chemistry, then medium

and cell lysates were collected for 5E1 immunoprecipitation and analysed by Shh Western blot. In both medium and cell lysates, YnC15-labeled Shh is much lower in hHhat KD group than in Hhat-Mut and control group, providing a consistent result with Fig. 4-6C. On the other hand, non-YnC15-labeled secreted Shh is more abundant in the hHhat KD cells but less abundant as cell associated form in cell lysates. Combining both observations suggests that un-palmitoylated Shh has lost ability in cell association, but instead is released to the medium.

4-2-6. PANC1 proliferation is Shh-dependent and inhibited by knocking down hHhat

To determine the effects of hHhat on PANC1 proliferation, hHhat RNAi KD combined with CFSE incorporation were used, followed by flow cytometry analysis, as described in Chapter 3 (practical assistance and advice was generously provided by Dr. Marek Cebecauer). In the assay, I set out to investigate whether PANC1 proliferation is Shh-dependent by treating cells with 5E1 inhibitory antibody for Shh. The results (Fig. 4-9F) have been discussed previously in Section 3-2-6 showing that PANC1 proliferation is Shh-dependent.

Interestingly, 7 days after transfection hHhat siRNA-treated cells had a significantly higher mean fluorescence intensity (MFI), with ~8 times greater CF fluorescence (>6000) compared to hHhat-Mut siRNA-transfected cells

(<1000) (Fig. 4-9F). In addition, the flow cytometry profile shows that there are two significant populations in the hHhat RNAi group but only one population in the hHhat-Mut RNAi group and untreated cells (Fig. 4-9A, D and E). These results indicate that siRNA-mediated KD of hHhat suppresses PANC1 cell proliferation substantially.

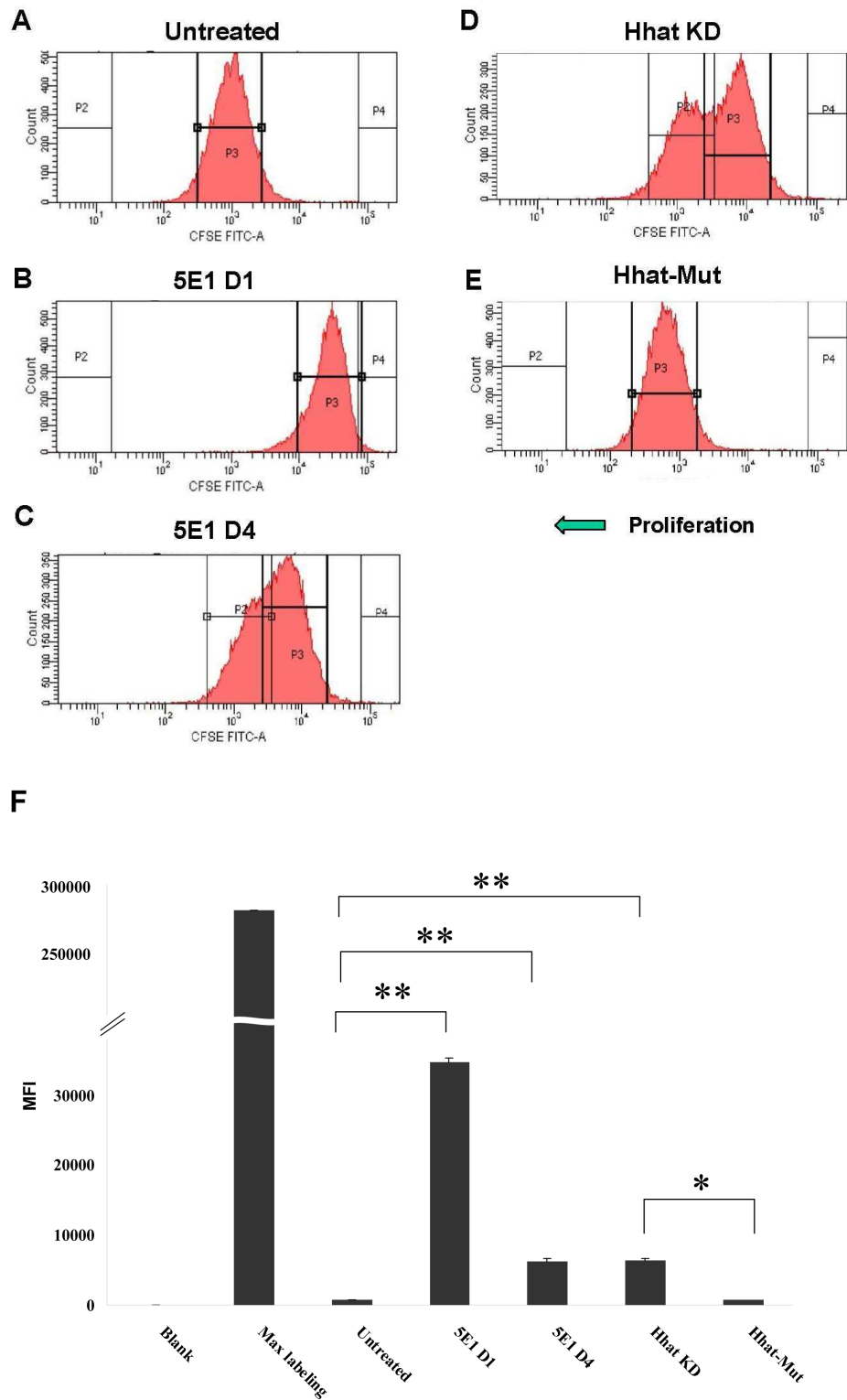


Figure 4-9. The role of hHhat in PANC1 cell proliferation. The histogram of flow cytometry is shown in A-E. The quantitative result is shown in F. 5E1

treatment on Day 1 (5E1 D1, B) was used to examine if PANC1 proliferation is Shh-dependent. 5E1 treatment on Day 4 (5E1 D4, C) was used to mimic RNAi KD *in vivo* (which is carried out for 72hr). The level of maximum labeling was observed by treating cells with CFSE just before flow cytometer assessment. To study the effect of hHhat in PANC1 proliferation, 8 days after RNAi transfection the cells were applied to the flow cytometer. In A-E, the X-axis shows the CF intensity. The green arrow shows the direction of peak shift observed when cells proliferate. In F, the Y-axis shows the CF intensity observed from 30,000 cells. The experiments were repeated three times in triplicate, and statistical significance was measured by using the two-tailed t test (*, $P < 0.00001$; **, $P < 0.0001$).

4-2-7. Knocking down hHhat inhibits PANC1 invasion

To investigate the contribution of hHhat to the invasive potential of PANC1 cells, I carried out invasion assays after Hhat knockdown. Initially, I showed that PANC1 invasion is dependent on Shh signalling using 5E1 treatment. The results (Fig. 4-10) have been discussed previously in Section 3-2-7, suggesting that the invasive ability of PANC1 cells is dependent on Shh signalling. Moreover, PANC1 cells were transfected with hHhat siRNA 72hr prior to the invasion assay. The reason why 72hr was chosen here is due to optimised condition for reduced Shh signalling in PANC1 cells caused by Hhat KD was observed on 72hr after siRNA transfection (Fig. 4-8 C and D). In this assay I observed a significant decrease in the number of invasive PANC1 cells (66 ± 7 cells/well), a level similar to that observed for the 5E1 treatment (Fig. 4-10). In contrast, Hhat-Mut siRNA control treatment gave similar levels as observed in untreated control cells (276 ± 40 cells/well). These results indicate that expression of hHhat was required for efficient PANC1 invasion. Importantly, direct cell growth assays showed that the doubling time for PANC1 cells is ~51hr. The four-fold reduction in invasion observed in 24hr cannot therefore be fully accounted for by inhibition of cell growth.

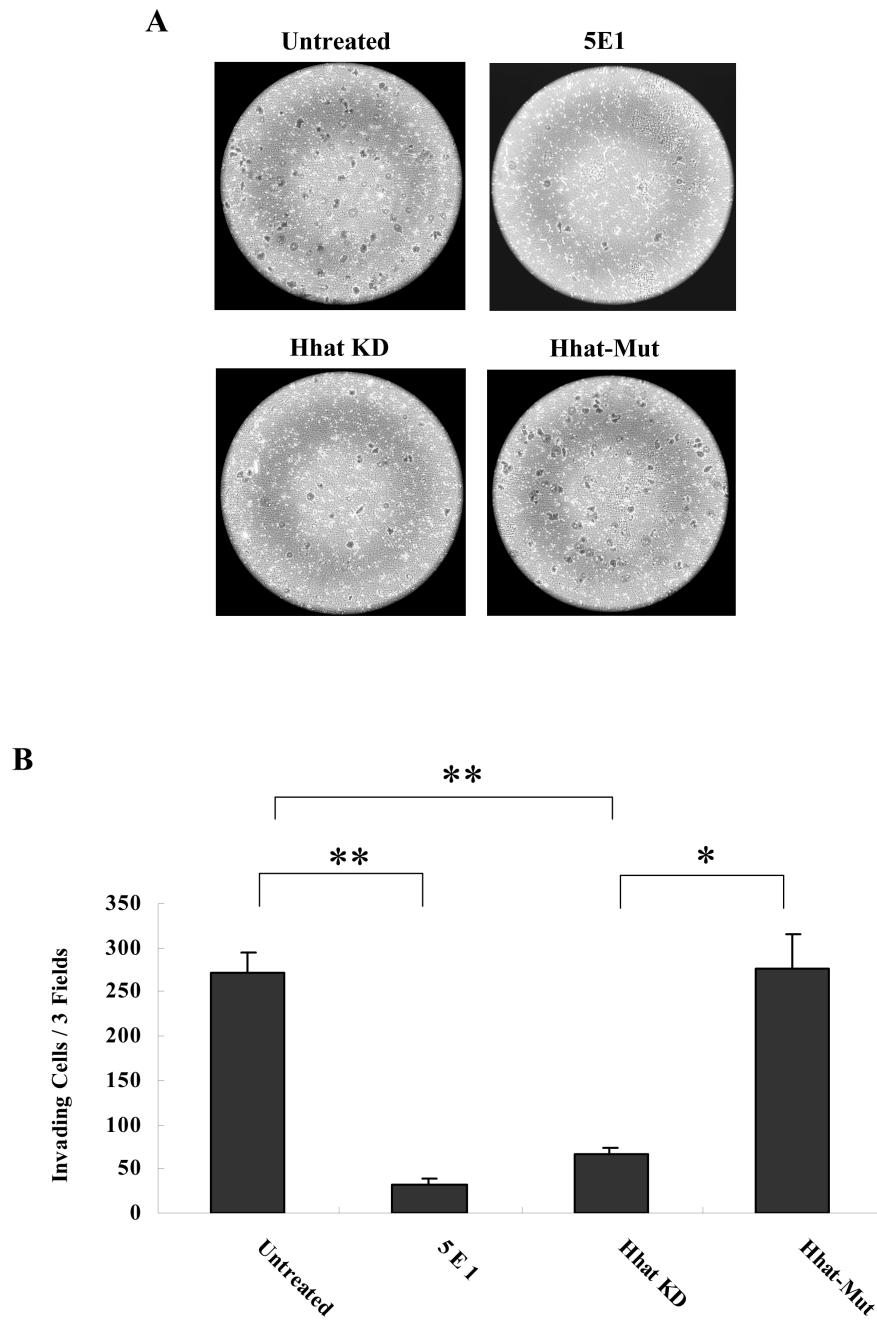


Figure 4-10. Biological function of hHhat in PANC1 cell invasion. I set out to determine whether PANC1 invasion is Shh dependent using 5E1 treatment. To study the effect of hHhat in PANC1 invasion, 72hr after RNAi transfection the cells were plated onto Matrigel invasion chambers for 24hr. Cells that had migrated from the upper to the lower side of the filter were photographed (A)

and counted with a light microscope (40 fields/filter, B). The experiments were repeated four times, and statistical significance was measured by using the two-tailed t test (*, $P < 0.001$; **, $P < 0.0001$).

4-2-8. Knocking down hHhat in PANC1 cells reduces endogenous Shh oligomerisation

To evaluate the role of hHhat in formation of Shh multimeric complexes, gel filtration followed by Shh dot blotting were carried out on PANC1 conditioned medium. To confirm the specificity of the Shh antibody, H160, which was to be used for dot blotting, it was tested against different culture media by Western blotting (Fig. 4-10A). The result shows that no detectable non-specific background was observed. In Shh dot blots (Fig. 4-11B), most secreted Shh in control PANC1 culture medium migrates as large multimeric complexes and only a small portion of Shh migrates as monomers ($2.6 \pm 1.2\%$, $P < 0.0001$), similarly to a previous study [188]. I then analysed whether hHhat was involved in this multimeric complex formation by hHhat siRNA treatment. Interestingly, in conditioned medium from hHhat siRNA-treated cells, significant quantities of Shh monomers were detected ($41.2 \pm 9.6\%$, $P < 0.0001$), nearly 20 times higher than in control medium, whereas Hhat-Mut siRNA-treated medium shows a similar pattern to control medium with only $2.1 \pm 1.5\%$ ($P < 0.0001$) monomer. These data suggest that KD of hHhat in PANC1 cells causes decreased Shh multimer secretion and further implies that palmitoylation of

Shh plays a positive role in Shh multimer formation.

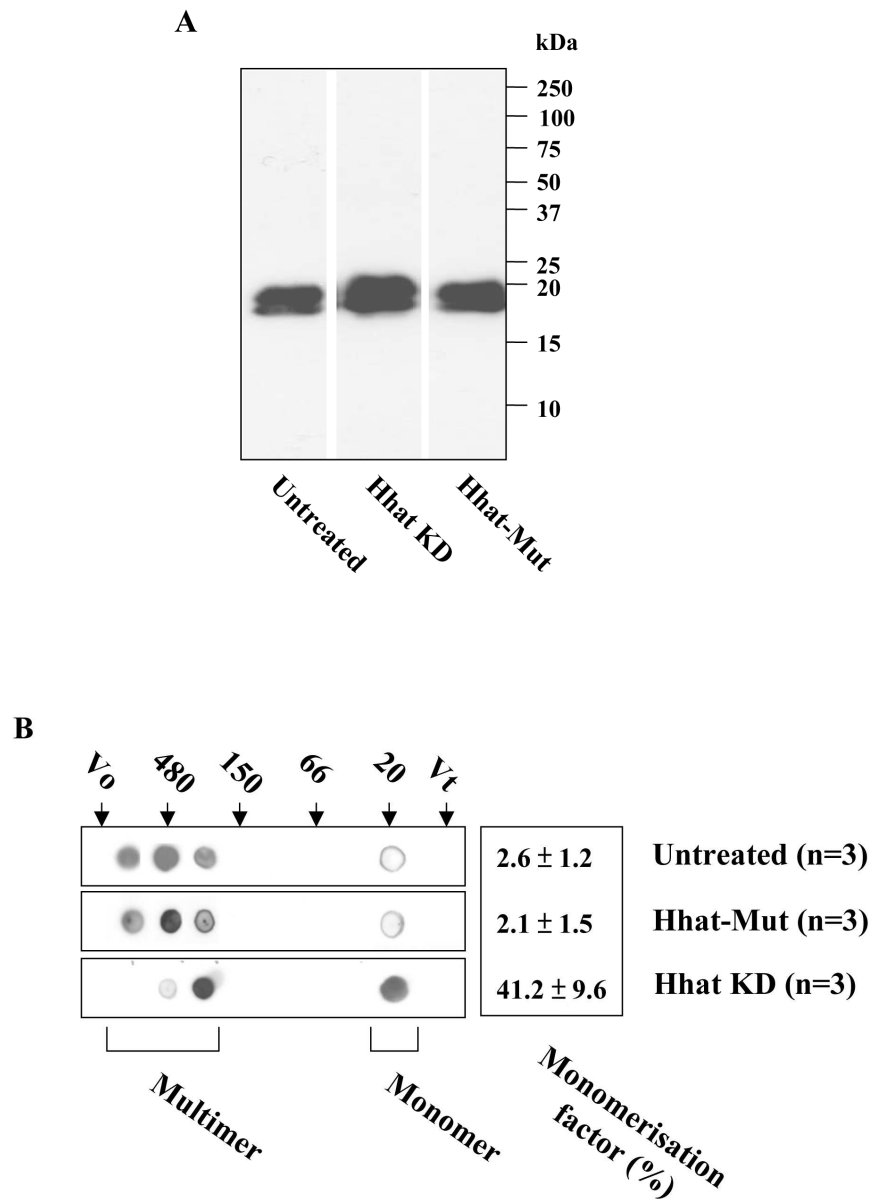


Figure 4-11. The role of hHhat in Shh multimeric complex formation. 72hr after RNAi transfection, the culture media were TCA precipitated and examined by Western blot of Shh (A). The result confirmed previous data (Fig. 4-8E), showing that un-palmitoylated Shh (Hhat KD group) is more abundant in the medium. In B, to examine the role of hHhat in Shh oligomerisation, 72hr

after RNAi transfection the media were subjected to gel filtration chromatography (Superdex200 10/300 GL column). After TCA precipitation, the fractions were probed by Shh dot blot (B). Monomerisation factor (%) = (Monomer / Monomer + Multimer) X 100%.

4-3. Discussion

Hedgehog proteins are unusual in being dually lipid-modified to be fully active. The lipidations are essential for efficient morphogen secretion, travel through the ECM, and accurate targeting on receiving cells. In this work, using an anti-peptide strategy we generated a human Hhat antibody which is the first antibody specific to Hhat. The specificity of this antibody was confirmed with immunoblotting using hHhat-EGFP- and mGup1-EGFP-transfected cells. In addition, this antibody was characterised by subcellular fractionation (S100/P100) showing that hHhat localised in the membrane fraction instead of the cytosolic fraction. Also, using this self-generated hHhat antibody combined with confocal microscopy, I found that in PANC1 cells endogenous hHhat localises in ER mainly but not in Golgi complex. Combined with a previous study showing that HA-tagged Hhat and Shh co-localised in the secretory pathway [12] in COS-1 cells, this suggests that palmitoylation of Shh occurs in the lumen of the ER. This is the first evidence showing the localisation of endogenous Hhat *in vivo*.

It has been reported that lipidations of Hh/Shh are essential for efficient morphogen secretion and appropriate travel through the ECM. Also, Hhat is identified as an enzyme directly and specifically required for the N-terminal addition of palmitate to Hh/Shh morphogens, and is believed to play an essential role in effective production of the Hh/Shh proteins [31,164]. Instead of using a traditional approach based on radiolabelled ^3H -palmitate incorporation, in this work I used an *in vitro* Shh palmitoylation using click-chemistry. The data presented in this study indicate that hHhat is responsible for Shh palmitoylation, showing a consistent result with studies in both *Drosophila melanogaster* and mammalian systems [164]. In addition, in this work I established conditions for effective RNAi KD of hHhat in PANC1 cells and found that it causes reduced signalling through the Shh pathway in PANC1 cells via examining Ptc and Gli1 expression, both target genes of Shh signalling. The KD efficacy was examined by Western blot of hHhat and the cell-based palmitoylation method. The protein levels of Ptc and Gli1 drop ~70% and ~50% respectively after hHhat KD, in agreement with mRNA reduction by >70% and >50%. The data presented herein provide the first direct evidence showing the functional role of Hhat in Shh signalling in a human model system.

In previous studies by Segal *et al.*, Shh^{Ala/Ala} and Shh^{Ala/-} mice show abnormal proliferation in cerebellar size, indicating suppressed neural stem/precursor cell proliferation in multiple locations in both the developing and adult CNS

caused by inefficient Shh signalling. In this study, due to the fact that pancreatic ductal adenocarcinoma (PDAC) is among the most aggressive of all cancers and is relatively resistant to medical treatments, we asked whether the decrease in palmitoylation of Shh is reflected in abnormal proliferation in human PDAC (PANC1) cells. In 5E1-treated PANC-1 cells, proliferation was strongly suppressed shown by highly maintained CF level observed, indicating Shh-dependent PANC1 proliferation. Furthermore, in hHhat KD cells PANC1 proliferation was approximately 3 times less than that in un-treated cells and Hhat-Mut-treated cells throughout the 8-days experiment. This decreased proliferation suggests that N-terminal palmitoylation of Shh promotes mitogenesis of PANC1 cells through Shh signalling. This is the first direct demonstration of reduced cell proliferation caused by Hhat KD in a mammalian system.

It has been reported that Hh/Shh signalling acts as a contributor to various tumour types e.g. pancreatic cancer, colon carcinoma, gastric cancer, small cell lung cancer (SCLC), prostate cancer, breast cancer and basal cell carcinoma [38,42,84,91-96,109,178,213]. Although it has been revealed that the Hh/Shh signal participates in cell migration and angiogenesis processes and is correlated with the severity of the associated tumour, especially the invasiveness of three human PDAC cell lines (AsPC-1, SUIT-2, and CFPAC-1) [212], to date no report gives a detailed description on the functional relevance of the Shh pathway to invasion of human pancreatic cancer PANC1 cells. In the present study, Shh signalling inhibition with 5E1, the inhibitory antibody of

Shh, reduces the invasion ability of PANC1 cells, indicating PANC1 that invasion is Shh-dependent. Moreover, the invasive ability of these cells was abolished by hHhat KD indicating that the palmitoylation of Shh plays a significant role in Shh signalling which results in Matrigel degradation and cell migration through the ECM. Here, I observed for the first time that Shh signalling is associated significantly with invasion of PANC1 cells. To sum up, hHhat is required for effective Shh signalling via adding a palmitate to the N-terminus of Shh, and is necessary for Shh-dependent PANC1 proliferation and invasion. Therefore, this study provides a novel perspective in which hHhat could be an effective therapeutic target in human pancreatic cancer treatment.

Changes in association to cell membranes and ability in multimeric complex formation provide two possible mechanisms by which palmitoylation of Shh could modulate signalling activity. In this work, the data from click-chemistry method reveal that un-palmitoylated Shh protein lost ability in cell association. On the other hand, the Shh oligomerisation assay provides strong evidence that palmitoylation plays a significant contribution in Shh multimeric complex formation. In addition to the evidence listed above, both blocked proliferation and invasion are in agreement with malfunction of Shh signalling caused by hHhat KD. Based on these observations, I hypothesise that reduced Shh palmitoylation causes the loss in cell retention and Shh oligomerisation, leading to inhibited cell proliferation and invasion.

CHAPTER 5

5. Summary, Final Discussion and Future Directions

5-1. Clinical treatments of PDAC

About PDAC. Pancreatic ductal adenocarcinoma (PDAC) remains one of the most difficult cancers to treat. About 95% of exocrine pancreatic cancers are adenocarcinomas. It is the commonest cancer affecting the exocrine pancreas. Without active treatment, metastatic pancreatic cancer has a median survival of 3–5 months and 6–10 months for locally advanced disease, which increases to around 11–15 months with resectional surgery [226]. Due to the late presentation and aggressive tumour biology of this disease, only a minority (10–15%) of patients can undergo potentially curative surgery.

Treatment of pancreatic cancers. PDAC is highly resistant to conventional methods of cytotoxic treatment and radiotherapy. Few chemotherapeutic agents have been shown to have reproducible response rates of more than 10%. 5-Fluorouracil (5FU) is an inhibitor of thymidylate synthetase (essential for synthesis of DNA nucleotides) and has been widely used in advanced pancreatic cancer, with a median survival of around 5–6 months [230]. Though radiotherapy has also been widely used for the treatment of pancreatic cancer, a recent meta-analysis demonstrated that chemoradiotherapy is better than radiotherapy alone and that there is no survival difference between chemoradiotherapy plus follow-on chemotherapy and chemotherapy alone [230]. A number of new agents (e.g. PA3, SWOG S0205, CALGB 80303, Avita,

GV1001 and TeloVac) have been developed from the molecular understanding of pancreatic cancer, which are now being assessed in large phase III trials in advanced pancreatic cancer [223,229]. Radical resection alone will result in a 5-year survival of only 10% owing to recurrence after surgery [231]. Nearly all patients develop metastatic disease, most commonly of the liver and peritoneum but also the lungs, and this may occur with or without local recurrence. To sum up, significant progress has been made in illuminating risk factors and key molecular events in pancreatic carcinogenesis, but still, at present only pancreatic resection can improve survival significantly. A further improvement in survival is achievable with adjuvant chemotherapy but not chemoradiotherapy. Though surgical approaches have become more standardised and are safer, with much improvement in both morbidity and mortality in specialised centres, surgery of pancreatic cancer can only be performed within a regional pancreas cancer. Therefore, there is a great need in development of earlier diagnosis, screening of high-risk groups and efficient treatment of pancreatic cancer.

Shh signalling in PDAC. Sonic hedgehog (Shh) signalling plays a crucial role in the initiation and growth of pancreatic cancer [103-106]. Cyclopamine inhibits the Shh pathway through direct interaction with Smoothened [214] and has now entered early clinical trial development. Recent studies suggest Shh may also act as a contributor to so-called pancreatic cancer stem cells [227]. Li *et al* have recently identified a CD44⁺CD24⁺ESA⁺ phenotype (0.2–0.8%) isolated from primary pancreatic cancer cells with a 100-fold increased

tumourgenic potential [227]. The existence of cancer stem cells is based on the hypothesis that the ability of a tumour to grow and propagate is dependent on a small subset of cells (< 5%) with special properties, which like normal stem cells, have a great potential for self-renewal and production of differentiated progeny [228].

5-2. The role of HSPGs in hShh signalling

To investigate the interaction between hShh and heparan sulfate proteoglycans (HSPGs) and clarify the biological function of hShh/HSPGs complexes in hShh signalling, in this study,

I took an alternative approach by generating mutant Shh proteins that cannot bind heparin properly. I used DNA mutagenesis (based on modelling of the hShh-heparin interaction by Dr. Barbara Mulloy, NIBSC, personal communication) and heparin affinity chromatography to determine key residues in hShh involved in heparin binding (K37/38 and K178). The above data showed for the first time that, besides the Cardin-Weintraub motif, K178 of hShh also plays a significant role in HSPGs binding.

Also, by detecting induced Alkaline Phosphatase activity in *C3H10T1/2* cells and hShh-inducible genes expression in PANC1 human PDAC cells via immunoblotting and semi-quantitative RT-PCR analysis as a readout; I examined the biological function of mutated hShhs (ShhNC24IIK37/38S, K178S and K37/38/178S) that cannot interact with heparin efficiently, and showed that they promoted lower signalling activity compared to wild-type

hShh and a control mutation (ShhNC24IIK74S). Together, these findings indicate that Shh-HSPGs interactions mediated by the Cardin-Weintraub motif and the novel residue 178K are significantly required for Shh signalling activity.

In addition, in this study employed 5E1 to block the Shh signalling pathway in PANC1 cells and to determine the effect of the pathway on proliferation and invasiveness of PANC1 cells, showing the first direct evidence that Shh signalling has a critical and central role in PANC1 cell growth and contributes to malignant transformation. Furthermore, I tested the effect of mutant hShh proteins on proliferation and invasion of PANC1 cells following endogenous hShh RNAi knockdown. These findings indicate that Shh-HSPGs interactions promote PANC1 cells oncogenic properties at multiple levels, including both proliferation and invasion; providing corresponsive results to previous studies, including: 1) Several Shh responsive genes; e.g. *Gli2*, *N-myc*, *Gli3*, *Cyclin D1*, *Cyclin D2* and *Bmi-1*, have been identified, including genes that are involved in proliferation and tissue patterning [196-202]. 2) *Cyclin D1*, *Cyclin D2* and *Bmi-1* gene targets that require Shh-HSPG interactions are significant in tumour stem cell biology [196,197]. 3) Shh-dependent cancer growth can be stimulated via glypican overexpression in several cases, e.g. prostate cancer, PDAC and rhabdomyosarcoma [203,204]. However, using a mammalian expression vector to preclude the possibility of misfolding of purified proteins would provide a better approach to examine the biological function of mutated Shh proteins which cannot bind to heparin properly.

Lastly, I used gel filtration chromatography to study the role of mutated hShhs in Shh multimeric complex formation. From the data obtained, here I hypothesise for the first time that K178 of Shh may play key role in Shh multimeric complex formation via binding with HSPGs. Supportive evidence is that in the 3D structure of Shh, K178 is located close to the HSPG-binding groove containing the Cardin-Weintraub motif (Dr. K. Grobe, personal communication).

These results must be considered with previous studies using mutations in the glypican Dally-like, mutations in the *Ext* gene family (the HSPG-synthesising enzymes) and truncation of the Cardin-Weintraub motif; which have suggested that HSPGs are critical for appropriate localisation of Hh proteins [55,138,139,206]. Here, I hypothesise a second potential mechanism to explain the effects of proteoglycans on Shh-induced proliferation and invasion in PANC1 cells - Shh-HSPGs interactions stimulate Shh signalling via forming stabilised multimeric complexes in ECM to facilitate stability and solubility of Shh proteins.

To sum up, the data provided in this study indicate that the gene expression pattern induced by Shh is modulated by proteoglycans of producing cells via forming stabilised multimeric complexes in ECM. Moreover, Shh target genes that are altered when cells are stimulated by different Shh proteins include many that have been implicated in proliferation and oncogenesis.

Specific achievements in the study are listed below:

- Determined key residues, including a known heparin binding motif K37/38 and a novel residue - K178, of Shh involved in heparin binding.
- Showed reduced biological function in *C3H10T1/2* model system stimulated by mutated Shh which cannot interact with heparin efficiently and verified the result with 5E1 (an inhibitory antibody specific to Shh) treatment.
- In PANC1 cells, Immunoblot and RT-PCR of Ptc and Gli1 showed a consistent result with *C3H10T1/2* system.
- wtShh and control mutant (K74S) can restore PANC1 division and invasion caused by Shh KD, but not triple mutant - ShhK37.38.178S.
- Used endogenous Shh KD to study the role of exogenous mutated Shh in Shh multimeric complex formation by gel filtration.

5-3. Characterisation of hHhat in PANC1 cells

To identify the intracellular localisation of hHhat in mammalian cells, I characterised an hHhat antibody prepared in the lab which is the first antibody specific to Hhat. By subcellular fractionation and Western blotting hHhat is found to be a membrane protein. Also, I confirmed the specificity of the antibody using a self-constructed hHhat-EGFP clone, and a control mGup1-EGFP clone using immunoblotting. In addition, I used the hHhat antibody to determine the intracellular localisation of hHhat in PANC1 cells by confocal microscopy and showed that hHhat localised in ER mainly but not in

Golgi apparatus. This results suggest that palmitoylation of Shh occurs in the lumen of the ER. I confirmed this using the hHhat-EGFP clone for fluorescence microscopy in transfected cells. Combined with the previous localisation study carried out by HA-tagged exogenous Hhat in COS-1 cells [12], the work here provides the first evidence for the localisation of endogenous hHhat *in vivo*.

Specific accomplishments in the study are listed below:

- Characterised hHhat antibody by subcellular fractionation and Western blotting.
- Confirmed the specificity of the antibody using a hHhat-EGFP clone, a control mGup1-EGFP clone and immunoblotting.
- Determined the intracellular localisation of hHhat in PANC1 cells by confocal microscopy and showed that hHhat co-localised in ER mainly but not in Golgi apparatus and verified via the hHhat-EGFP clone for fluorescence microscopy in transfected cells.

5-4. The role of hHhat in hShh signalling

To illuminate the biological function of palmitoylation of hShh in production of active hShh and in the formation of hShh multimeric complex, I optimised hHhat RNAi KD in PANC1 cells and confirmed by an *in vitro* palmitoylation assay, click-chemistry method, showing a consistent result with studies in both *Drosophila melanogaster* and mammalian systems in which Hhat is required for the N-terminal addition of palmitate to Hh/Shh morphogens [12,31].

Next, using semi-quantitative RT-PCR and immunoblot analyses, I examined the role of hHhat in production of active hShh, which causes decreased signalling through the Shh pathway in PANC1 cells, providing the first direct evidence for the functional role of Hhat in Shh signalling in a human model system. Here, Hhat's role as a positive mediator in Shh signalling may be caused by its potency in increasing cell retention. To confirm this possibility, I investigated the effect of the addition of palmitate to hShh on its secretion from producing cells (or attachment to receiving cells) by comparing cell-associated hShh in cells that have hHhat silenced by RNAi oligonucleotides with control cells. The click-chemistry assay and Shh immunoblotting data both suggest that un-palmitoylated Shh protein lost ability in cell association, providing consistent results with previous findings in *Drosophila* [31]. Palmitoylation of Shh can improve its ability to associate to cell membranes, providing a possible mechanism by which palmitoylation of Shh could modulate the signalling activity.

In *Drosophila*, RNAi-mediated Hhat KD reduces N-terminal palmitoylation of Hh *in vivo* and causes a reduction in cell association as indicated by a significant increase in release of Hh protein into the culture medium [31]. Furthermore, in my study using hHhat RNAi KD, gel filtration of high molecular weight complexes of hShh and immunoblotting of hShh I characterised the role of palmitoylation of hShh in hShh multimeric complex formation. The results provide strong evidence that the palmitoylation is involved in Shh multimeric complex formation. This result also gives another possible mechanism by

which palmitoylation of Shh could modulate signalling activity, by changing its ability in multimeric complex formation and increasing proteins stability in ECM.

It has been shown previously that the Hh/Shh signal participates in cell migration and angiogenesis processes and is correlated with the severity of the associated tumour, especially the invasiveness of three human PDAC cell lines (AsPC-1, SUI-2, and CFPAC-1) [212]. However, no report has given a detailed description on the functional relevance of the invasion and proliferation of human PDAC PANC1 cells to the Shh pathway. In this study, 5E1-treatment of PANC1 cells indicates that invasion and proliferation is Shh-dependent. Notably, I investigated the effect of Hhat KD on PANC1 proliferation and invasion showing that knocking down Hhat represses PANC1 proliferation and invasion. These data suggest that N-terminal palmitoylation of Shh promotes mitogenesis of PANC1 cells through Shh signalling, and give the first direct demonstration of reduced cell proliferation and invasion caused by Hhat KD in a mammalian system. To sum up, hHhat is required for effective Shh signalling via adding a palmitate to the N-terminus of Shh, and is necessary for Shh-dependent PANC1 proliferation and invasion.

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Combining the above observations, I hypothesise that reduced Shh palmitoylation causes the loss in cell retention and Shh oligomerisation, leading to inhibited cell proliferation and invasion in PANC1 cells.

Specific achievements in this project are listed as following:

- Established conditions for effective knockdown of hHhat – causes decreased signalling through the Shh pathway in PANC1 cells.
- Knocking down Hhat inhibits PANC1 proliferation and invasion.
- Studied the role of Shh palmitoylation in Shh multimeric complex formation and cell retention.

5-5. Future perspective

Dysregulation of Hh/Shh signalling pathway leads to carcinogenesis as mentioned in Section 1-1-4, and Hh/Shh signalling components are pharmacogenomics targets. Furthermore, loss-of-function mutations in negative regulators of the pathway, including Ptc1 and SuFu, have been associated with tumorigenesis, indicating the potential of inhibitors of Hh signalling act as tumour suppressors, as follows. 1) The Veratrum alkaloid cyclopamine functions as a Hh/Shh signalling inhibitor via binding to Smo and inducing Smo misfolding within the ER [214]. 2) The synthetic cyclopamine-derivative cyclopamine-KAAD, with increased potency for Hh/Shh signalling inhibition, is being applied as an anti-cancer drug clinically [215]. 3) SANT1-4 and Cur61414 are also small-molecule compounds which inhibit Hh/Shh signalling through binding to Smo [8,216]. 4) Other small-molecule modulators, inhibiting the signalling pathway mostly at the level of Smo, were also identified, supporting that Hh/Shh signalling pathway can be blocked at different levels [217]. On the other hand, to increase the potency for

inhibition in Hh/Shh signalling and to decrease the occurrence of drug resistance, a cocktail of Hh/Shh inhibitors could be developed as novel therapeutics for human cancer treatment. In this work, my studies provide a firm basis for understanding the functional roles of hShh palmitoylation and its interactions with HSPGs, and provide proof-of-principle for targeting these aspects of hShh biology in tumour cell therapeutics, specifically in the pancreatic carcinoma context.

On one hand, since hHhat is required for effective Shh signalling via adding a palmitate to the N-terminus of Shh, and is necessary for Shh-dependent PANC1 proliferation and invasion, this study provides a novel perspective in which hHhat could be an effective therapeutic target in human pancreatic cancer treatment. On the other hand, the HSPGs seem to play a key role in regulating Shh stability, movement and reception through the ECM via forming multimeric complexes, leading to the following questions. Is the degree of sulphation on the HS GAG chains involved? Is cholesterol on Shh also significant in this multimeric complex formation? Do unidentified enzymes together with HSPGs regulate signalling? As a summary, a current model demonstrating the regulation of Shh signalling in mammalian cells is presented in Fig. 5-1. In the future, it would be interesting to find out the mechanism of this multimeric complex formation, and specific HSPG(s) involved in Shh signalling in tumour cells, which would provide further insight into the workings of the signalling pathway and the exploration of the therapeutic potential of Shh pathway inhibitors. Also, it would be helpful to build up gene-expression

profiles of human tumours in determining which cell types have activated Hh/Shh signalling and therefore be useful in predicting the outcome of potential treatments with Hh/Shh pathway inhibitors.

To sum up, the findings in this work are exciting as inhibition of the pathway could lead to novel therapeutic approaches to primary and metastatic tumours. This is particularly important as the high frequency of metastasis in PDAC during early stages of the disease, often before diagnosis, is one of the complications that contribute to low survival rates [218].

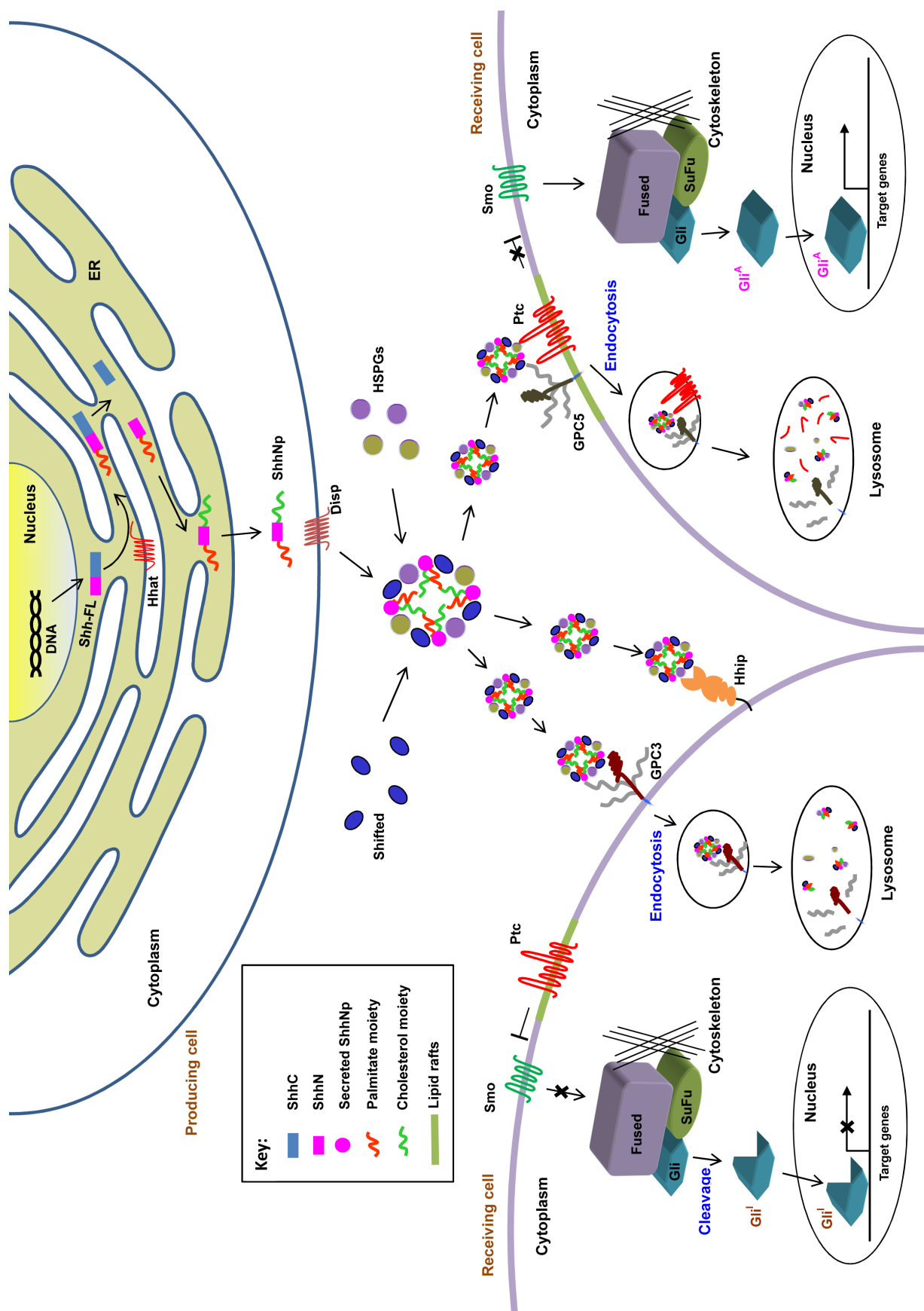


Figure 5-1. A model for Shh signalling regulation in mammalian cells. In the Shh producing cell (upper), Shh is synthesised as a full-length precursor (Shh-FL) in the ER. After its signal peptide is removed co-translationally, a palmitate is added to the N-terminus of Shh via Hhat in the ER lumen, followed by autocleavage and C-terminal cholesterol modification to form a fully modified Shh protein (ShhNp). After trafficking from internal membranes to plasma membrane, the ShhNp secretes to the ECM and forms multimers with HSPGs and Shifted (the Wnt inhibitory factor homologue [146]. Dispatched (Disp) is believed to be involved in this secretion. On the signal-activated receiving cell (right bottom), Shh-HSPGs-Shifted multimers bind to Ptc and Glypican-5 (GPC5) [232] leading to internalisation of the complex and activation of Smo. On the contrary, in the signal-inhibited receiving cell (left bottom), Ptc, Hhip and Glypican-3 (GPC3) compete for binding to Shh. When Shh binds to GPC3 it is followed by GPI-dependent endocytosis, as a consequence Shh is removed from cell surface, prevented from binding to Ptc leading to downregulated signal transduction.

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