STUDIES OF SMOOTHENED IN HEDGEHOG SIGNALING PATHWAY

APPROVED BY SUPERVISORY COMMITTEE

Mentor: Jin Jiang, Ph.D.

Committee Chairman: Helmut Krämer, Ph.D.

Committee Member: Melanie Cobb, Ph.D.

Committee Member: James Chen, Ph.D.

**This dissertation is dedicated to my husband,**

**who is always there for me.**

# STUDIES OF SMOOTHENED IN HEDGEHOG SIGNALING PATHWAY

By

Chao Tong

DISSERTATION

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STUDIES OF SMOOTHENED IN HEDGEHOG SIGNALING PATHWAY

By Chao Tong Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2006

Supervising Professor: Jin Jiang Ph.D.

The Hedgehog (Hh) family of morphogens controls cell growth and patterning in both vertebrates and invertebrates. Malfunction of Hh signaling has been implicated in numerous human disorders. As the Hh signal transducer, the seven-transmembrane protein Smoothened (Smo) is highly regulated. It is still a mystery how Smo transduces graded Hh signal to downstream components.

Although Smo shares some structural similarity with G protein coupled receptors (GPCR), there is little evidence that G proteins are involved in Hh signal transduction in physiological settings. A kinesin like protein Costal2 (Cos2) and a serine/threonine kinase Fused (Fu) form complexes with the transcription factor Cubitus-interrupts (Ci), which is essential for Hh signal transduction. However, how Smo transduces Hh signal to this complex is still not clear. In this study, we found that Smo interacts with Cos2-Fu complex through its C-terminal tail, which is essential for the Hh pathway activation.

In response to Hh, Smo is phosphorylated and accumulated on the cell surface. However, the kinases responsible for Hh induced Smo phosphorylation are still unknown. It is also not clear whether phosphorylation regulates Smo activity or not. In this study, I found that protein kinase A (PKA) and casein kinase I (CKI) regulate Smo cell surface accumulation and activity in response to Hh. PKA and CKI phosphorylate Smo directly at multiple sites which form three clusters in Smo C-terminal tail. In cooperation with Jianhang, we found that phosphorylation deficient forms of Smo failed to accumulate on the cell surface and were unable to transduce Hh signal. By contrast, phosphorylation mimicking forms of Smo have increased cell surface accumulation and constitutive activity. In addition, we also found the levels of Smo cell surface accumulation and activity correlate with its phosphorylation levels, suggesting that the graded Smo activity may be regulated by differential phosphorylation of its C-terminal tail.

Furthermore, I have identified multiple Arginine clusters in Smo the C-terminal tail that negatively regulate Smo activity by preventing Smo cell surface accumulation and keeping Smo C-terminal tail in a closed inactive conformation maintained by intramolecular electrostatic interactions. I have also found that the number of arginine clusters is reversely correlated with Smo cell-surface expression and activity. I also provided evidence that phosphorylation

antagonizes the negative effects of the Arginines by neutralizing the positive charges they carry, which lets Smo C-terminal tail adopts an open and active conformation and promotes Smo cell surface accumulation. Based on these data, we proposed that multiple arginine clusters provide a way to finetune Smo activity in response to different Hh levels by differentially phosphorylating Smo C-terminal tail.

This study also showed that Gprk2, a G protein coupled receptor kinase (GRK), plays a positive role in regulating Hh signalling. I provided evidence that Gprk2 interacts with Smo C-tail. Furthermore, I identified a new CKI phosphorylation cluster that appears to be critical for Smo endocytosis and activation.

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LIST OF ABBREVIASIONS

AKAPs - A-kinase-anchoring proteins

A/P - Anterior/ Posterior

APC - adenomatous polyposis coli

Arm - Armadillo

*botv* - brother of tout velu

cAMP - cyclic AMP

CBP - CREB-Binding Protein

Ci - Cubitus-interrupts

CIP - calf intestine phosphatase

CKI - casein kinase I

*collier* - col

Cos2 - Costal 2

CRD - cysteine rich domain

Cul - Cullin

*Dhh* or Dhh - Desert hedgehog

*Disp* or Disp - Dispatched

*dlp* or Dlp - dally- like

*dpp* or Dpp - decapentaplegic

Dvl - dishevelled

ECM - extracellular matrix

EGF - Epidermal Growth Factor

*en* or En - engrailed

ER - endoplasmic reticulum

EXT - Exostosin

FRET- fluorescent resonance energy transfer

Fu - Fused

Fz - Frizzile

GAG - glycosaminoglycans

GAS-1 - growth arrest-specific 1

GBP - GSK3 binding protein

GIRP- G protein-activated inwardly rectifying potassium

GPCR - G protein coupled receptor

GRK - G protein coupled receptor kinase

GSK3 - glycogen synthase kinase 3

Hh - Hedgehog

HIB - Hh induced BTB domain containing protein

Hip-1 - Hedgehog-interacting protein 1

HS - heparin sulfate

HSPGs - heparin sulfate proteoglycans

IFT - intraflagellar transport

*Ihh* or Ihh - Indian hedgehog

KD - Kilo Dalton

LMP - leptomycine

LV - longitudinal vein

NLS - nuclear localization signal

PBS - Phosphate buffered saline

PBT - Phosphate buffered saline with Triton X-100

PCP - planar cell polarity

PKA - protein kinase A

Ptc - Patched

RNAi - RNA interference

RND - resistance, nodulation, division family proteins

SAID- Smo auto-inhibitory domain

Sgg - Shaggy

*shf* - shifted

*shh* or Shh - Sonic hedgehog

Skn - Skinny hedgehog

Smo - Smoothened

*sotv* - sister of tout velu

SSD - sterol-sensing domain

Sufu - Suppressor of Fused

β-TrCP - β-Transducin repeat Containing Protein

*ttv* - tout velu

Ub - ubiquitin

Wg - Wingless

WIF-1 - Wnt inhibitory factor-1

ZPA - zone of polarizing activity

# CHAPTER ONE

# Introduction and Literature Review

## Hedgehog Signaling Pathway Overview

The Hh signalling pathway directs many aspects of metazoan development. As a morphogen, Hh provides an inducive signal to promote cell proliferation and differentiation in various pattern formation processes including limb formation, left-right asymmetry determination, and neural tube differentiation (Ingham and McMahon, 2001). In addition, Hh signal also directs formation and maintenance of stem- or precursor- cell populations in a variety of postnatal tissues (Lai et al., 2003; Reya et al., 2001; Zhang and Kalderon, 2001). Due to the essential embryonic and postnatal roles of Hh signaling pathway, the malfunction of this pathway is involved in numerous human birth defects and cancers (Taipale and Beachy, 2001). One effect of reduced Hh signalling in human embryos is cyclopia, a syndrome in which embryos only develop one eye (Chiang et al., 1996; McMahon et al., 2003). In contrast, increased Hh signalling leads to cancers in various adult tissues and organs such as skin, cerebellum, pancreas, and digest tracts (Pasca di Magliano and Hebrok, 2003).

Due to its essential role in human health, the Hh signalling pathway has been extensively studied since *hh* gene was first identified in *Drosophila* by genetic screen (Nusslein-Volhard and Wieschaus, 1980). Subsequent studies in several model organisms showed that the molecular mechanisms used to produce, receive and transduce the Hh signal are largely conserved from *Drosophila* to mammals (Ingham and McMahon, 2001).

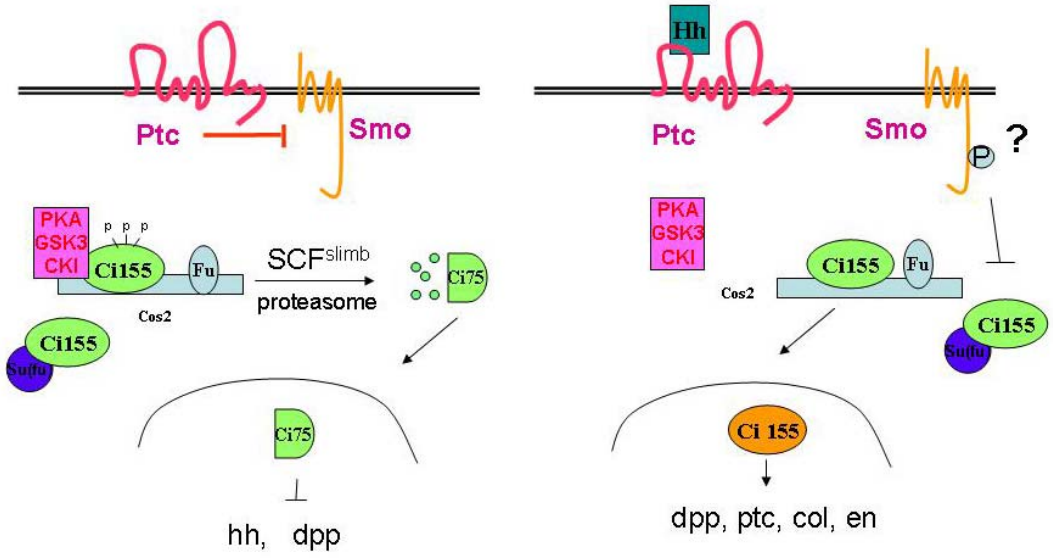



Fig. 1.1- Hedgehog signal pathway in *Drosophila.* The transcription factor Ci155 forms complex with either Cos2 and Fu or with Sufu in the cytoplasm. In the absence of Hh (left panel), Ptc inhibits the activity of Smo. Cos2 recruits PKA, CKI, and GSK to phosphorylate Ci155 and promote subsequent processing mediated by $SCF^{slimb}$ complex in the proteasome pathway. The processing of Ci155 results in the production of the repressor form of Ci: Ci75, which goes into nuclei and inhibits downstream gene such as *dpp* expression. Once Hh binds to Ptc (right panel), the inhibition on Smo is released and Smo is phosphorylated and accumulated on cell surface. Hh blocks Ci processing by dissociating kinases from Cos2 and release Sufu's inhibition on Ci155 and activate Ci155. The active form of Ci155 induces expression of target genes such as *dpp*, *ptc*, *col* and *en*.

The outline of *Drosophila* Hh signalling pathway is shown in Fig. 1.1. Hh is a secreted molecule whose reception system consists of two multispan trans-membrane

proteins, Patched (Ptc) and Smo (Ingham and McMahon, 2001). In the absence of Hh, Ptc blocks Hh signaling pathway by inhibiting Smo. As a result, the downstream transcription factor Ci is proteolytically processed via SCF E3 ubiquitin ligase containing the F-box protein Slimb, to form a truncated protein, Ci75, which represses the expression of downstream genes such as *decapentaplegic* (*dpp*) (Aza-Blanc et al., 1997; Jiang and Struhl, 1998; Methot and Basler, 1999). Once Hh binds to Ptc, the inhibition of Smo is released. Smo signals downstream to prevent Ci processing and stimulate the transcriptional activator activity of the full length Ci (Ci155), which subsequently induces the expression of downstream genes including *dpp*, *ptc* and *engrailed* (*en*)(Alexandre et al., 1996). In the cytosol, the kinesin-like protein Cos2 interacts with Ci, the serine-threonine kinase Fu, and the tumor suppressor protein called Suppressor of Fused (Sufu) (Monnier et al., 1998; Robbins et al., 1997; Sisson et al., 1997). These proteins form multiple protein complexes that regulate Ci processing and subcellular localization (Chen et al., 1999a; Methot and Basler, 2000; Wang et al., 2000; Wang and Jiang, 2004; Wang and Holmgren, 2000). In the absence of Hh, Ci is hyperphosphorylated by PKA, CKI and glycogen synthase kinase 3 (GSK3) (Jia et al., 2002; Jia et al., 2005; Price and Kalderon, 2002). These three kinases also form complexes with Cos2 and Ci, which facilitates Ci phosphorylation and its subsequent processing (Zhang et al., 2005).

## Hh Production and Modification

In *Drosophila*, there is only one *hh* gene, which was first identified as a segment polarity gene whose mutations lead to segment polarity defects in *Drosophila* embryos (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). In embryos, *hh* is expressed in the posterior part of each segment and acts as an on-off

switch to induce Wingless (Wg) expression in the adjacent cells and direct *Drosophila* embryo segmentation (Ingham, 1993). In *Drosophila* wing imaginal discs, Hh works as a short range morphogen and low levels of Hh induce *dpp* expression, intermediate levels of Hh induce *collier* (*col*) and *ptc* expression, and high levels of Hh induce *en* expression (Strigini and Cohen, 1997; Vervoort et al., 1999). Unlike the *Drosophila*, there are several Hh related genes in vertebrate species (Ingham and McMahon, 2001). In mammals, there are three members of *hh* family: *Sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*), and *Desert hedgehog* (*Dhh*)(Echelard et al., 1993). *Dhh* is most closely related to *Drosophila hh* and mainly regulates spermatogenesis, while *Ihh* and *Shh* are close to each other and play important roles in embryonic development. Strikingly, *Shh* is expressed in several key signalling centers: the notochord, the floor plate, and the zone of polarizing activity (ZPA), and regulates patterning of the neural tube and the limb (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994).

To fulfill their important biological functions, Hh proteins need to be cleaved and modified by lipid in the producing cells before they are secreted. Although initially most modifications were identified in *Drosophila*, the overall processing and modification mechanisms are conserved in vertebrates (Ingham and McMahon, 2001; Mann and Beachy, 2004).

*Drosophila hh* gene encodes a 45 KD secrete peptide which enters the secretory pathway and undergo autoprocessing (Bumcrot et al., 1995; Lee et al., 1994). The C-terminal portion of Hh possesses an autoprocessing activity that catalyzed an intramolecular cleavage of the full length Hh (Lee et al., 1994; Porter et al., 1995). The resulting Hh-N terminal fragment becomes a mature signalling molecule after a

cholesterol moiety is covalently linked to its C-termini and a palmitic acid is added to its most N-terminal cysteine residue (Mann and Beachy, 2004). In *Drosophila*, the palmitoylation of Hh-N does not depend on cholesterylation (Chamoun et al., 2001). However, in mammals the cholesterylation seems to be necessary for palmitoylation (Pepinsky et al., 1998). An acycltransferase named Skinny hedgehog (Skn) (also known as Cmn, Rasp, and Slightless) catalyses the palmitoylation process and is essential for the proper Hh signalling (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001). The addition of cholesterol and palmitic acid increases the hydrophobicity of Hh proteins and may let the mature Hh attach to the membrane of the sending cells, which prevents free diffusion of Hh and provides a platform to regulate Hh gradient formation (Gallet et al., 2003; Porter et al., 1996). In addition, these modifications may also let Hh adopt a right conformation and possess optimal signalling activities (Mann and Beachy, 2004).

## Hh Secretion and Transport

As a morphogen, Hh not only signals to the adjacent cells but also forms a concentration gradient and provides developmental cues to the receiving cells. In *Drosophila* wing imaginal discs, Hh could travel a dozen cell diameters (~ 20 um) and control the expression of different target genes as a function of its concentration (Strigini and Cohen, 1997). In vertebrate neural tube, Shh gradiant could span over ~200 um and control several cell fate determination processes (Jacob and Briscoe, 2003). However, the lipid modifications attach Hh to the sending cell membrane and decrease its mobility. How do sending cells release and spread lipid modified Hh to the receiving cells?

The identification of *Dispathed* (*Disp*) provides an answer to this question. The *Disp* gene is only required in Hh producing cells. In *Disp* mutants, the production and processing of Hh are normal. However, Hh only signals to the cells adjacent to the Hh producing cells and no long-range signalling event is observed, suggesting *Disp* is required for Hh release from the sending cells (Burke et al., 1999). There are two *Disp* homologs in mammals. Mouse genetic studies showed *Disp1* plays similar roles as *Drosophila Disp* in delivering lipid modified Shh (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002b; Tian et al., 2004). Like Hh receptor Ptc (we will talk about it later), Disp protein is a twelve transmembrane protein with a sterol-sensing domain (SSD) that is often involved in the regulation of protein subcellular localization and vesicle trafficking in cholesterol homeostasis or cholesterol linked signaling (Kuwabara and Labouesse, 2002), suggesting that vesicle trafficking may be important for Disp functions. Based on the sequence similarity, Ptc and Disp are closely related to bacterial membrane transporters known as resistance, nodulation, division (RND) family proteins (McKeegan et al., 2003). Mutating the conserved residues required for RND activity in Disp disrupts the biological function of Disp, suggesting that Disp may be functionally related to the transporter (Ma et al., 2002b). However, the precise mechanism of how Disp releases Hh is still not clear.

After Hh proteins are released by the sending cells, they travel many cell diameters to form a concentration gradient. The mechanism responsible for Hh spreading is still elusive. Although some morphogens are transported by transcytosis (Tabata and Takei, 2004), the experimental data argue against this model as a key mechanism for Hh transport. Indeed, Hh is mainly observed extracellularly in the receiving tissues and the

transport of Hh is facilitated instead of blocked by inhibiting endocytosis (Gallet and Therond, 2005; Han et al., 2004a; Torroja et al., 2004). Currently, the most favorite model is that Hh is diffusing actively through the extracellular matrix (ECM). More and more evidence in both *Drosophila* and vertebrates showed that several heparin sulfate proteoglycans (HSPGs) actively participate in Hh transport through the ECM (Kramer et al., 2003). HSPGs are extracellular molecules with a protein core to which heparin sulfate (HS) glycosaminoglycans (GAG) chain are attached. In *Drosophila*, the genes *dally* and *dally- like* (*dlp*) encoding glypicans and *trol* endoding a perlecan core protein are required for Hh signalling (Lin, 2004). In *dlp* mutant embryo, no Hh signaling is detected out of its expression zone, indicating that *dlp* is involved in Hh movement (Han et al., 2004b). However, the study in *Drosophila* culture cells indicated that *dlp* is only required for Hh reception but not transport (Desbordes and Sanson, 2003; Lum et al., 2003a). These controversial results may be due to the difference between the systems used by different groups. In addition, *dally* and *dlp* are required to stabilize Hh in the posterior of drosophila wing imaginal discs.

In addition to *dlp* and *dally*, a few genes belonging to Exostosin (EXT) family which is involved in HSPGs biosythesis, modification and processing are also showed to regulate Hh signalling. Three genes identified in Drosophila: *tout velu*(*ttv*), *sister of tout velu* (*sotv*), and *brother of tout velu* (*botv*) are important for HSGAG polymerization and HS chain initiation and elongation (Bellaiche et al., 1998; Bornemann et al., 2004; Takei et al., 2004). The genes *sulfateless*, *sugarless*, *fringe connection*, *slalom,* and *notum* all belong to EXT family and they play different roles in HSPCs modification and processing (Esko and Selleck, 2002; Giraldez et al., 2002; Goto et al., 2001; Lin, 2004).

In the mutant clones of those genes, the cells still can receive Hh signal but they cannot propagate it and the movement of Hh is impaired, indicating HSPGs is required for Hh transport. Interestingly, Hh proteins are not stable in EXT mutant clones (Bornemann et al., 2004; Tabata and Takei, 2004). These data suggest that HSPGs may have dual roles in regulating Hh signaling.

Recently, a Drosophila gene named *shifted* (*shf*) was shown to regulate Hh transport and stability in the same manner as HSPGs (Glise et al., 2005; Gorfinkiel et al., 2005). The genetic studies show that adult wings have dramatic *hh* mutant phenotype in *shf* mutant flies. In addition, *shf* also has genetic interactions with Hh pathway components *ci* and *fu*, indicating *shf* is involved in Hh signalling pathway. Further studies show that the transport of lipid modified Hh is compromised and the stability of Hh is reduced in the Hh expression cells in *shf* mutant flies. Shifted protein is a *Drosophila* ortholog of vertebrate Wnt inhibitory factor-1 (WIF-1)(Hsieh et al., 1999). They both have a signal sequence, a WIF domain, and five EGF repeats. However, their functions are quite different in different organisms. In mammals, WIF-1 sequesters Wnt protein and inhibits Wnt signalling. However, Shifted has no effect on Wingless (Wg) signalling in *Drosophila* but only regulates Hh signalling, indicating there are some fundamental differences that make Shifted and WIF-1 to recognize different molecules and function differently. Consistent with this, overexpression of mammalian WIF-1 cannot rescue *shf* mutant in *Drosophila*, instead, it causes Wg signalling defects. In addition, Shifted was found to interact with Hh but not Wg protein. Interestingly, HSPGs component Dlp also interacts with Shifted. Since HSPGs could interact with many signaling molecules, Shifted may function as a cofactor to help HSPGs recognize and bind specifically to Hh.

It still needs to be tested whether WIF-1 is required by Hh signalling pathway in vertebrate. On the other hand, several extracellular modulators of Shh have been identified in vertebrate such as: Hedgehog-interacting protein 1(Hip-1) (Chuang and McMahon, 1999), growth arrest-specific 1(GAS-1)(Lee et al., 2001), and Megalin (McCarthy et al., 2002). These factors all have negative effects on Hh signaling activity, largely due to their interfering with ligand/ receptor interaction.

## The Reception System of Hh: Ptc and Smo

After it is released from the sending cells and transported in the ECM, Hh finally meets its receptor, Ptc (Ingham and McMahon, 2001). Unlike the canonical ligand-receptor relationship which ligand activates receptor, Hh inhibits its receptor Ptc and releases Ptc's inhibition of Smo (Hooper and Scott, 1989; Ingham et al., 1991; Nakano et al., 1989; van den Heuvel and Ingham, 1996). Although Ptc seems to catalytically inhibit Smo activity, the molecular mechanism is still elusive (Taipale et al., 2002). Ptc is not only the receptor of Hh, it is also a target gene of the Hh pathway. This negative feedback loop is a hallmark of Hh signaling pathway (Capdevila and Guerrero, 1994; Goodrich et al., 1996; Ingham and Fietz, 1995; Marigo et al., 1996; Tabata and Kornberg, 1994).

### Ptc

Ptc is a twelve transmembrane protein closely related to the bacteria proton-driven transmembrane transporters known as RND proteins (Taipale et al., 2002). Ptc and RND proteins share similar structure of twelve transmembrane domains with large extra-cellular loops situated between the first and second, and the seventh and eighth transmembrane domains. In bacteria, the RND proteins function as a pump to remove antibiotics, toxic organic compounds, and metal iron from cells (McKeegan et al., 2003).

The function of Ptc is impaired by mutating the residues which are conserved in and required for the function of the bacteria transporter, consistent with Ptc functioning as a transmembrane transporter (Taipale et al., 2002). Several small molecules have been identified that inhibit or activate vertebrate Smo (Chen et al., 2002a), which raises an interesting possibility that Ptc may pump out small molecule which activates Smo or pump in Smo antagonist which inhibits Smo activity. However, until now small molecules that function in the physical conditions have not been identified and how Ptc inhibits Smo is still waiting to be tested.

As a morphogen, different Hh concentrations determine different cell fates; however, how different concentrations of Hh are perceived and interpreted by cells is still a mystery. A traditional model of morphogen interpreting is that the absolute number of active receptors tranduces information of different morphogen concentrations into the cells. In this case, the inactive receptors have little, if any effects on signal transduction. Another model is that both active and inactive receptors are important for cells to interpret ligand concentration. The recent study of Casali and Struhl (Casali and Struhl, 2004) suggests that the second model is favored in the case of Hh concentration reading. To test those two concentration reading models, they expressed different levels of a constitutive active form of Ptc, $Ptc^{\Delta Loop2}$, which cannot bind to Hh but is still able to inhibit Smo activity in wing imaginal discs and assayed their effects on the Hh pathway activation. Interestingly, they found that low levels of $Ptc^{\Delta Loop2}$ only had mild inhibitory effects on the expression of Hh target gene such as *dpp* in wild type cells; however, the same levels of $Ptc^{\Delta Loop2}$ in cell without endogenous *ptc* strongly inhibited Hh pathway activation. These data indicate that the Hh pathway activation does not solely depend on

the active form (unliganded form) of Ptc. By expressing $Ptc^{\Delta Loop2}$ alone or together with a wild type Ptc in posterior wing imaginal discs which express high levels of Hh but lack endogenous form of Ptc, they found that the liganded form (inactive) Ptc titrated off the inhibitory effects of the unliganded form (active) Ptc. These data suggest that the ratio of liganded (inactive) to unliganded (active) Ptc is critical for Hh pathway activation. To determine the exact ratio for Hh pathway activation, they expressed constitutive ligand bound form of Ptc (Ptc-Hh) and $Ptc^{\Delta Loop2}$ at different ratio and assayed the pathway activation. When the ratio of Ptc-Hh to $Ptc^{\Delta Loop2}$ is 3:1 or greater, Hh pathway is initiated. When the ration drop to 3:2 or lower, Hh pathway is blocked. Although the ratio does not provide a clear mechanism of how Ptc functions, it suggests that multiple Ptc molecules may form a functional complex. Consistent with this, a recent study showed that Ptc C terminal domain formed a trimer (Lu et al., 2006).

There is only one *ptc* gene in *Drosophila*. In wing imaginal discs, *ptc* is expressed in anterior compartment and is up-regulated at the Anterior/ Posterior (A/P) boundary by Hh signaling. It has been found that Hh could travel further and induce downstream gene expression in the anterior cells immediately anterior to the *smo* mutant clones that abut the A/P boundary because of the loss of *ptc* expression in those *smo* clones (Chen and Struhl, 1996). Interestingly, ectopically inducing high level Ptc expression in P compartment near A/P boundary blocks Hh spreading to anterior compartment. These data suggest that Ptc could restrict the spread of Hh. Since Hh up-regulates Ptc expression at the A/P boundary, this regulation provides a self-limiting mechanism by which Hh restrict its own range of action.

Two *ptc* genes, *ptc1* and *ptc2* have been identified in vertebrates. *Ptc1* appears to be the major receptor during embryonic development (Goodrich et al., 1997; Wolff et al., 2003). Human Ptc2 protein has a 54% overall identity to Ptc1 (Carpenter et al., 1998). They share similar structural organization but they are different in both N- and C-terminal regions. The expression pattern of Ptc1 and Ptc2 are different during development of epidermis, indicating they may have different functions (Motoyama et al., 1998; Smyth et al., 1999). The function of Ptc2 is still unclear. In zebra fish, Ptc2 mutants show mild phenotype (Koudijs et al., 2005). Rare Ptc2 mutations could be found in medulloblastoma and basal cell carcinoma (Smyth et al., 1999).

**Smo**

Smo is a seven transmembrane protein similar to G protein coupled receptors (GPCR)(Alcedo et al., 1996; van den Heuvel and Ingham, 1996). However, no evidence was shown that G protein activation is necessary for activating Hh signaling pathway (DeCamp et al., 2000; Kasai et al., 2004; Riobo et al., 2006). How Smo activates Hh pathway is still an open question.

As a Frizzled (Fz) family member, Smo also has a cysteine rich domain (CRD), which is essential for Fz binding to its ligand Wnt. However, Smo's CRD neither binds to Wnt nor to Hh or other known ligand (Nusse, 2003). But the CRD of Smo is essential for Smo's activity in *Drosophila*. The deletion of CRD from *Drosophila* Smo abolishes Smo activity (Alcedo et al., 2000; Nakano et al., 2004). Surprisingly, the CRD is not required for vertebrate Smo activity (Murone et al., 1999; Taipale et al., 2002), indicating different regulatory mechanisms are applied between *Drosophila* and vertebrates. It is possible that

CRD may bind to some small molecules that either activates or inhibits Smo activity. The other possibility is that CRD is critical for maintaining Smo structure.

Both the extracellular domain and the transmembrane domain are quite conserved between *Drosophila* Smo and vertebrate Smo (Huangfu and Anderson, 2006). The C-terminal tail is the most diverse part of Smo across different species. *Drosophila* Smo has an extraordinary long C-terminal tail that is essential for its activity.

In *Drosophila*, with the stimulation of Hh, Smo is hyperphosphorylated (Denef et al., 2000); however, the kinases responsible for Hh induced Smo phosphorylation are still unknown. It is also unclear whether Smo phosphorylation is important for its function.

In *Drosophila*, Smo is stabilized and accumulated on cell surface in response to Hh (Denef et al., 2000); however, the mechanism of Smo cell surface accumulation is still elusive. In the absence of Hh, Smo is targeted to the degradation pathway and mainly found in lysosomes (Nakano et al., 2004). While in vertebrates, Smo is accumulated on cilia in response to Shh (Corbit et al., 2005). A point mutation destroying Smo cilia localization compromises Smo activity, suggesting that cilia localization is essential for Smo activation. Since many proteins involved in cilia formation also regulate Hh pathway activity in vertebrates (Huangfu and Anderson, 2005; Liu et al., 2005), the cilia may provide a platform for Hh signal transduction. Although cilia accumulation of Smo is important, Smo endocytosis also appears to be essential for Smo activity in vertebrates. It has been shown that GRK2 phosphorylated Smo and recruited β-arresting 2, which induced Smo endocytosis. The loss of function studies in zebrafish embryo (Wilbanks et al., 2004) indicate that function of β-arresting 2 but not β-arresting 1 is necessary for Smo to transduce Hh signal. However, β-arresting 2 knock out mice are alive and have no Hh

pathway related defects. It is possible that there are redundancy issues. Strikingly, β-arresting 1, 2 double knock out mice are embryonic lethal, but whether there are *shh* mutant like phenotypes is still waiting to be investigated.

**How does Ptc inhibit Smo activity?**

Even without Hh stimulation, Smo is activated and induces downstream gene expression in the *ptc* mutant clones, indicating Smo is constitutively active and its activity is inhibited by Ptc in the absence of Hh. How Ptc inhibits Smo activity and how Hh release this inhibition are still mysteries.

Ptc may inhibit Smo activity by regulating Smo trafficking. It has been reported that Ptc colocalizes with Smo in the absence of Hh. With Hh stimulation, Ptc and Smo separate with each other and Smo goes to recycling pathway instead of degradation pathway (Incardona et al., 2002). However, controversial results regarding of Ptc and Smo trafficking have been reported and more investigation need to be done (Torroja et al., 2004; Zhu et al., 2003).

Since Smo is phosphorylated in response to Hh, Ptc may inhibit Smo by regulating Smo modification. However, whether phosphorylation of Smo is critical to its function is still waiting to be test.

Currently, the most favorite model is the pump model (Taipale et al., 2002). In this model, Ptc pumps out Smo agonist in absence of Hh. Once Hh bind to Ptc, the transporter function of Ptc is abolished, which facilitates Smo agonist to bind and activate Smo. The transporter like structure of Ptc and the point mutation analysis support that Ptc may function like a pump. The identification of small molecules such as cyclopamine which binds to Smo and inhibits Smo activity supports the idea that Smo is regulated by some

cholesterol like small molecules (Chen et al., 2002a). However, so far there is no small molecule has been found that functions as a Smo ligand under physiological conditions. Although the identification of small molecule is difficult, the synthesis or modification enzymes for the small molecule could be identified using traditional genetic method.

## Cos2: the dual role of a kinesin like protein

Cos2 was first found as a negative regulator of Hh signaling pathway. *cos2* mutant embryos without both maternal and zygotic contributions die with a cuticle pattern similar to that of *ptc* homozygote mutant embryos (Grau and Simpson, 1987). In adults, *cos2* mutations cause up-regulation of *dpp* expression (Capdevila and Guerrero, 1994) and duplication of the appendages (Grau and Simpson, 1987; Whittle, 1976), indicating Cos2 negatively regulates Hh signal transduction. However, in addition to these negative effects, Cos2 seems to play a positive role in the Hh pathway (Wang et al., 2000; Wang and Holmgren, 2000). In *cos2* mutant clones, abutting the A/P boundary, the expression of highest threshold target gene *en* is compromised. These data suggest that Cos2 activity is essential for Hh signal to turn on high threshold downstream gene expression. However, the exact mechanism of how Cos2 functions as a positive regulator is still elusive.

*cos2* gene encodes a kinesin-like protein (Robbins et al., 1997; Sisson et al., 1997). The N-terminal region is predicted to form globular structure as other kinesin family proteins and it preserves several conserved motifs for microtubule binding. Within the Cos2 motor domain, there is a conserved P-loop which is necessary for ATP binding and hydrolysis to promote kinesin protein translocation on the microtubule. Deletion of the P-loop or mutating the key amino acid in the P-Loop resulted in a dominant negative Cos2 that derepressed *dpp* but not *ptc* (Ho et al., 2005). The C-terminal region of Cos2 also has

a globular structure that may serve as a "cargo" binding domain. The central region of Cos2 contains 36 heptad repeats that are predicted to mediate Cos2 dimerization through a coil-coil structure (Woolfson and Alber, 1995).

Cos2 forms a complex with the serine-threonine kinase Fu, transcriptional factor Ci, and a novel protein Sufu (Robbins et al., 1997; Sisson et al., 1997). In response to Hh, both Fu and Cos2 are phosphorylated (Therond et al., 1996) and the whole complex falls off from the microtubule (Robbins et al., 1997). The C-terminal part of Fu is essential for Cos2-Fu interaction and this interaction may facilitate Fu to phosphorylate Cos2 directly in response to Hh (Robbins et al., 1997). The kinase assay carried in a baculovirus system showed that Fu phosphorylates Cos2 at Ser572 and Ser931(Nybakken et al., 2002). However, there is no evidence showing that mutations of those phosphorylation sites affect Cos2 activity in vivo. The biological function of Cos2 phosphorylation is still waiting to be investigated.

Cos2 interacts with two distinct domains of full length Ci, an amino terminal domain and a carboxyl terminal domain (Wang et al., 2000). These interactions trap Ci inside of the cytoplasm and prevent Ci nuclear translocation. In addition to cytoplasm retention of Ci, Cos2 also seemed to regulate Ci processing (Zhang et al., 2005). It has been shown that Ci is phosphorylated by PKA, GSK3 and CKI and the phosphorylation of Ci triggers Ci processing (Jia et al., 2002; Price and Kalderon, 2002). In *cos2* mutant wing imaginal discs, the level of Ci is elevated and the phosphorylation of Ci is compromised. Interestingly, both the N- and C- terminal regions of Cos2 associate with those kinases directly, indicating that Cos2 may recruit those kinases to phosphorylate Ci

in the absence of Hh. Hh causes dissociation of kinases from Cos2, thus blocking Ci phosphorylation and processing (Zhang et al., 2005).

Although Cos2 is a very important regulator in *Drosophila* Hh signaling pathway, the orthologs of Cos2 in mammalians do not function similarly (Varjosalo et al., 2006). Two orthologs of Cos2, kif7 and kif27 have been found in mouse. They share 39% and 37% sequence similarity with *Drosophila* Cos2 respectively. However, they do not appear to regulate Shh signaling in mouse cells (Varjosalo et al., 2006). It is possible that mammalians use a different mechanism to regulate Hh signal pathway and the Cos2 like activity is absent. The other possibility is the corresponding factor of Cos2 in mammalian system has not been identified yet. The sequence similarity comparison may not work in this case. More vigorous experiments need to be done to further confirm the function of mammalian Cos2 orthologs.

## Fused (Fu): What's the substrate?

In *Drosophila*, high threshold Hh responses require a Serine/Threonine protein kinase: Fused (Preat et al., 1993; Therond et al., 1993). *fu* mutants are embryonic lethal, displaying a segment polarity defect similar to *hh* mutants (Forbes et al., 1993; Nusslein-Volhard and Wieschaus, 1980). Maternal copies of *fu* mRNA could rescue the embryonic lethality of *fu* mutants and allow the mutant embryos to develop into adults. In adult wings of *fu* mutants, the longitudinal veins 3 and 4 (LV3 and LV4) are fused (Fausto-sterling, A, 1978). Additionally, the wings of *fu* flies have a posterior extension of the double row of marginal bristles, indicative of loss of *en* expression (Hidalgo, 1994; Sanchez-Herrero et al., 1996).

Fu protein consists of a kinase domain and a C-terminal regulatory domain. In *Drosophila* Hh signaling pathway, the kinase activity of Fu is required for most of its functions. However, the substrates of Fu have not been well-defined so far (Alves et al., 1998; Ohlmeyer and Kalderon, 1998; Therond et al., 1996). There is indirect evidence that Fu may phosphorylate Cos2 and Su(fu)(Ho et al., 2005; Lum et al., 2003b; Nybakken et al., 2002). But the biological relevance of these findings is still waiting to be tested. The C-terminal part of Fu (Fu-C) is essential for Fu-Cos2 interaction and therefore is required for proper Hh signal transduction (Robbins et al., 1997). Overexpression of Fu-C shows the same phenotypes as *fu* mutant, probably due to competing for Cos2 with endogenous Fu (Ascano et al., 2002).

It has been shown that Fu-C and Fu-N interact with each other and this intra-molecular interaction is essential for Fu activity *in vivo*. Over-expression of Fu-C in a *fu* mutant background that harbors mutations in the C-terminal part of Fu restores the LV3-LV4 intervine space. However, this rescue is still Hh dependent. In addition, the wing margin bristles still develop abnormally, indicating Fu-C and Fu-N interaction is not sufficient to produce hyperactive form of Ci (Ascano and Robbins, 2004).

In response to Hh, Fu is highly phosphorylated (Therond et al., 1996). However, the kinases responsible for phosphorylating Fu are still unknown. The data from *in vitro* kinase assay indicated that Fu is capable to auto-phosphorylate itself (Nybakken et al., 2002). However, it is likely that other kinases are involved in Hh induced Fu phosphorylation. The biological roles of Fu phosphorylation are still unknown. The identification of Fu phosphorylation sites may provide insight into the function of Fu

phosphorylation. It is possible that phosphorylation may change Fu conformation and facilitate its substrate recognition.

In zebrafish, one Fu homolog has been identified: stk36 (Wolff et al., 2003). Like *Drosophila* Fu, Stk36 is also required for responding to high levels of Hh. The mouse Fu homolog Stk36 is broadly expressed in embryonic tissues (Chen et al., 2005). In contrast to *Drosophila*, it has been reported that the kinase activity of vertebrate Fu is not essential for its activity in Hh signaling pathway. More intriguingly, mice lacking Stk36 activity survived after birth and showed no Hh related phenotypes (Chen et al., 2005; Merchant et al., 2005). It is possible that other kinases distantly related to Fu may play redundant roles in mice or Fu activity is not necessary for Hh signal transduction in mammals.

**Sufu: a negative regulator in Hh signaling pathway.**

*Sufu* was identified in an effort to find dominant suppressors of *fused* gene mutation (Pham et al., 1995; Preat, 1992; Preat et al., 1993). Interestingly, amorphic Sufu mutants are viable and without any obvious morphological defects. However, in the absence of Sufu, all *fu* related phenotypes are suppressed, such as segmentation defects, fused wings, and ovary tumors (Pham et al., 1995; Preat, 1992; Preat et al., 1993). There are three different classes of *fu* mutations. For class 0 and class I mutants, the *fu* mutant phenotypes are completely suppressed in $fu^{0}$, $Sufu^{-}$, and $fu^{1}$, $Sufu^{-}$ females. However, for class II mutants, a $cos2^{-}$ like phenotype was observed concomitant with suppression of *fu* phenotypes (Pham et al., 1995). Interestingly, the class I mutants mainly affect the kinase domain of Fu, while the class II mutations contain truncations in Fu-C terminal region (Ascano et al., 2002). In addition, *Sufu* geneticly interacts with *ptc*, *slimb*, *PKA* and *cos2* mutations (Ohlmeyer and Kalderon, 1998; Wang et al., 2000).

Sufu protein contains a PEST domain but no significant homologies to other known proteins (Pham et al., 1995). The PEST domain is involved in protein instability (Rechsteiner and Rogers, 1996). It is unknown whether protein turn-over is important for Sufu function *in vivo*. Sufu forms a tetra-complex with Ci, Cos2 and Fu (Methot and Basler, 2000; Stegman et al., 2000). Yeast two hybrid data suggest that Sufu interacts with Ci and Fu directly (Monnier et al., 1998), which is likely to be important for tethering Ci in the cytosol and inhibiting Ci activity. However, recent study suggests that Ci-Su(Fu) interaction facilitates Sufu to transport to nuclei and inhibit Ci activity probably by recruiting transcription co-repressor (Sisson et al., 2006).

In response to Hh, Sufu is phosphorylated. The phosphorylation sites haven't been mapped yet and the kinases responsible for Sufu phosphorylation are still unknown. Interestingly, loss of Fu activity results in the reduction of Sufu phosphorylation (Ho et al., 2005; Lum et al., 2003b), indicating that Fu activity is required for Sufu phosphorylation, either directly or indirectly. It is possible that phosphorylation changes Sufu conformation and blocks its interaction with Ci and therefore activates Ci. The identification of the phosphorylation sites on Sufu may help to answer these questions.

The vertebrate homolog of Sufu is highly conserved and has been extensively studied. Importantly, *Sufu* mutations have been found in human medulloblastoma and prostate cancer (Pasca di Magliano and Hebrok, 2003; Taylor et al., 2002), indicating *Sufu* is a tumor suppressor gene. In contrast to the weak phenotypes of *Drosophila Sufu* mutants, loss of *Sufu* resulted in complete activation of Hh signaling pathway in both mouse embryo (Cooper et al., 2005) and cultured mouse cell lines (Varjosalo et al., 2006), implying the divergence occurred during the evolution. Two distinct domains, Sufu-N

and Sufu-C, in human Sufu were identified to bind to Gli1 and are required for Gli1 cytoplasmic tethering and repression (Merchant et al., 2004). Mutation in the $Asp^{159}$ of Sufu, which is highly conserved from fly to mammal, compromised cytoplamic tethering and repression of Gli1 without obvious disruption of Sufu-Gli1 interaction (Barnfield et al., 2005). These results indicat that distinct mechanisms might be applied to bind, tether and repress Gli1. Interestingly, mouse Sufu was found to interacted with Sap18, a component of mSin3 and histone deacetylase complex, and the deacetylase activity seems to be necessary for repressing Gli transcription activity (Cheng and Bishop, 2002). This finding adds another layer of regulation on Gli transcription activity. However, *in vivo* study of Sap18 is still lacking.

**Ci: complex regulation of a transcription factor.**

Ci is a transcription factor mediating Hh target gene expression (Orenic et al., 1990). Several layers of regulatory processes control Ci activity in response to Hh: 1) proteolytic processing, 2) cytoplasmic/nuclear translocation, and 3) activation. These processes are all highly regulated, which may endow Ci the ability to gradually respond to different levels of Hh (Ingham and McMahon, 2001; Jia and Jiang, 2006; Lum and Beachy, 2004).

Ci protein contains five Zn-finger domains that mediate DNA binding (Forbes et al., 1993; Pavletich et al., 1993). The C-terminal region of Ci interacts with a transcription co-activator CREB-Binding Protein (CBP), which is essential for Hh responsive gene expression (Akimaru et al., 1997; Methot and Basler, 1999). In *Drosophila* wing imaginal discs, Ci is present in several different states: truncate form of Ci (Ci75, CiR), full length form of Ci (Ci155) (Aza-Blanc et al., 1997; Methot and Basler, 1999), and hyperactive form of Ci (Ci*) (Ohlmeyer and Kalderon, 1998) as shown in Fig. 1.2. Interestingly,

different states of Ci regulate the expression of different subsets of Hh responsive genes (Methot and Basler, 1999; Wang and Holmgren, 1999) (Fig.1.2).

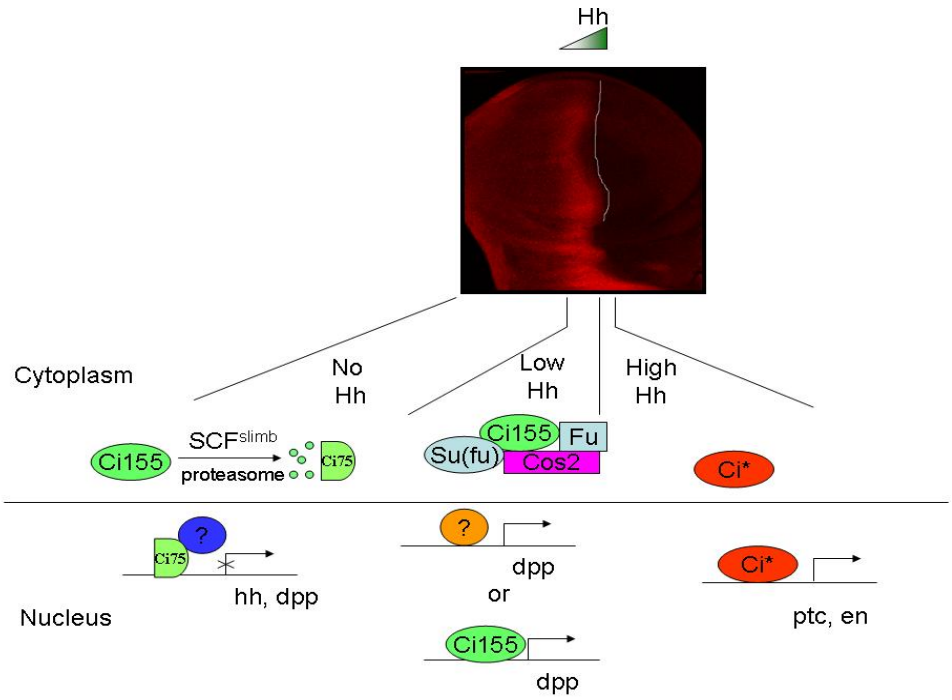



Fig. 1.2 - Different states of Ci regulate the expression of different subsets of Hh responsive genes in response to Hh gradient. Full length Ci staining shown roughly three Ci expression domain in anterior cells reflecting no Hh, low Hh and high Hh conditions (upper panel). A/P boundary was marked by the vertical line. Far away from A/P boundary, Ci is processed by $SCF^{Slimb}$ to form the repressor form Ci75 which inhibits Hh target gene such as *dpp* expression presumably by occupying the enhancer sequence but lacking activation domain or by recruiting other transcription co-repressor. In the low Hh zone near A/P boundary, processing of Ci is inhibiteded. However, Ci155 is still repressed by Sufu. *dpp* expression is turned on by other transcription factor or small amount of active form Ci. In the cells just abutting A/P boundary, the high Hh expression zone, Ci escapes from the inhibition of Sufu and mature to a labile hyperactive form Ci*, which activates high threshold downstream genes such as *ptc* and *en* expression.

Away from the A/P boundary**,** there is no Hh and full length Ci is processed to the truncated form Ci75 that localizes to the nucleus and functions as a transcription repressor (Aza-Blanc et al., 1997; Hepker et al., 1997), presumably due to its binding to DNA without recruiting CBP. However, it is also possible that Ci75 recruits some

transcription co-repressor to repress target genes such as *dpp* expression. *dpp* transcription is regulated by both activator and repressor forms of Ci. Blocking Ci processing is not sufficient to fully activate *dpp* expression (Methot and Basler, 1999).

Near the A/P boundary, Hh prevents Ci from being processed and active form of Ci induces d*pp* and *ptc* expression. Just abutting to the A/P boundary, there is a narrow strip of anterior cells with low level hyperactive form of Ci* (Ohlmeyer and Kalderon, 1998). Ci* is a labile transcription factor that induces high threshold Hh target gene *en* expression. Fu activity is necessary for Ci* formation and Sufu antagonizes this process. Loss of Fu activity results in loss of *en* expression with concomitant stabilization of Ci. It has been shown Ci* instability is not due to its processing. However, the biochemical property of Ci* is still an enigma. It is possible that Ci* has additional post-translation modifications that enhance its activity but reduce its stability at the same time.

Ci processing is highly regulated. An F-box containing protein Slimb/ β-TRCP is required to generate the repressor form of Ci (Jiang and Struhl, 1998). In addition, proteasome inhibitors block Ci processing, indicating Ci is processed through a proteasome dependent mechanism (Aza-Blanc et al., 1997; Chen et al., 1999a). Remarkably, Slimb together with Skp1, Cullin1 (Cul1) and Rox/Rbx1 RING finger protein forms an Ub ligase (E3) complex SCF (Deshaies, 1999). Consistent with the role of $SCF^{Slimb}$ in Ci processing, the subsequent studies also showed that Cul1 is also involved in proteolytic processing of Ci (Ou et al., 2002). Interestingly, Slimb and its vertebrate homolog β-TRCP are also involved in proteolysis of β-catenin/Arm and IκB and regulate Wnt/Wg and NF-κB/Dorsal pathways (Hart et al., 1999; Kitagawa et al., 1999; Latres et al., 1999; Spencer et al., 1999; Winston et al., 1999; Yaron et al., 1998).

F-box proteins tent to recognize the phosphorylated substrates. Consistently, Slimb/ β-TRCP binding motifs in β-catenin, IκB, and other Slimb/ β-TRCP substrates conform a consensus sequence: $D_pSGXX_pS$. Surprisingly, this consensus sequence is not present in Ci. However, Ci indeed is phosphorylated in the absence of Hh, which is essential for its processing (Chen et al., 1999b; Jia et al., 2002; Price and Kalderon, 1999, 2002; Wang et al., 1999). PKA was first identified as a negative component of the Hh pathway required for Ci processing. Loss of function of PKA catalytic subunit resulted in constitutive Hh signaling. The subsequent study showed that PKA phosphorylated a C-terminal region at multiple sites that are essential for Ci processing. Several recent studies showed that GSK3 (Sgg) and CKI are also required for Ci processing(Jia et al., 2002; Price and Kalderon, 2002). Interestingly, PKA, GSK3, and CKI phosphorylation sites form three clusters in Ci C-terminal part. Recent studies from our lab and others showed that phosphorylation of those clusters confered binding to Slimb/ β-TRCP (Jia et al., 2005; Smelkinson and Kalderon, 2006). Although the interaction between the single phosphorylation motif and Slimb/ β-TRCP is weak, the multiple phosphorylation clusters convert the weak interaction into a strong one which is sufficient to mediate Ci ubiquitination and processing. Since kinases such as GSK3 are involved in numerous signaling pathways, that rises an interesting question of how Ci phosphorylation is regulated in the Hh signaling specific manner. Remarkably, Cos2 was found to be necessary for Ci phosphorylation and processing (Zhang et al., 2005). Studies from our lab have shown that Cos2 interacted with PKA, GSK3 and CKI directly, which is necessary for efficient Ci phosphorylation and processing. In response to Hh, these

kinases are dissociated from Cos2, and leading to inhibition of Ci phosphorylation and processing.

In addition to Ci processing, the amount of nuclear Ci appears to be tightly controlled by a nuclear/cytosol shuttling process (Chen et al., 1999a; Wang et al., 2000; Wang and Holmgren, 1999, 2000). Ci has a functional nuclear localization signal (NLS) but is primarily localized in the cytoplasm throughout its expression domain in wing discs. A region (aa675-860) just carboxyl terminal to the Ci cleavage sites was first identified as a cytoplasm localization signal (Aza-Blanc et al., 1997). Later on, it was found to contain a nuclear export signal which promotes Ci nuclear export to maintain Ci in a unstimulated state (Chen et al., 1999a). In the wing discs treated with leptomycine (LMP), a potent nuclear export inhibitor, nuclear accumulation of Ci was observed in a strip of cells near the A/P boundary but not in other Ci expression domain even after Ci processing is blocked by proteasome inhibitor, suggesting Hh stimulation is essential for Ci nuclear shuttling (Chen et al., 1999a). With Hh stimulation, Ci is transported into nucleus, however, this process is masked by rapid nuclear export. With the presence of LMP, Ci is constitutively localized in the nucleus in *ptc* mutant discs. However, in the *smo* mutant clones, even if there is Hh, Ci is still maintained in cytoplasm (Wang and Holmgren, 2000). These data suggest that Hh signal transduction is required for Ci nuclear transport. Interestingly, both Cos2 and Su(fu) bind to Ci and tether Ci in the cytosol (Wang et al., 2000).

It has been established that the activation of Ci is a separate event from preventing Ci processing and Ci nuclear trafficking (Wang and Holmgren, 2000). The expression of the highest threshold Hh target gene *en* requires Ci activation. Both Sufu and PKA

prevent Ci activation by unknown mechanisms independent of preventing Ci nuclear trafficking. However, the biochemical properties of Ci* is unclear so far. The only clue is that Ci* becomes more labile than the full length Ci (Ohlmeyer and Kalderon, 1998). Our lab's recent finding showed that a Hh induced BTB domain containing protein (HIB) mediates Ci* degradation and decrease Hh signaling by a negative feedback mechanism (Zhang et al., 2006). HIB forms a complex with Cul3, a scaffold protein for ubiquitin ligases, and mediates Ci ubiquitination and degradation by proteasome pathway (Zhang et al., 2006). Since the level of Ci* is increased in HIB mutant cells, we may be able to study whether Ci* has specific modifications by blocking HIB mediated degradation. It is possible that some specific modifications endow Ci interacting with specific sets of transcription co-activators or escaping sufu mediated repression.

**Divergence of Hh signal transduction between *Drosophila* and vertebrate.**

Although the framework of Hh signaling pathway is conserved between *Drosophila* and vertebrate, more and more studies suggest that divergences exist (Huangfu and Anderson, 2006; Ingham and McMahon, 2001). The most obvious difference between *Drosophila* and vertebrate is that many components have been duplicated during evolution (Ingham and McMahon, 2001). For example, there are only one *hh* gene, one *ptc* gene, and one *ci* gene in *Drosophila*. However, there are three *hh* genes, two *ptc* genes and three *gli* genes in mouse (Ingham and McMahon, 2001). In some cases, the duplication events reflect the increased complexity of Hh function in different organs and different development stages. Consistent with this, different Hh homologs have distinct expression patterns and play different roles in development of different organs. For example, Shh and Ihh play important roles in embryonic development (Chiang et al.,

1996; St-Jacques et al., 1999; Zhang et al., 2001), while Dhh regulates spermatogenesis (Bitgood et al., 1996). In other cases, the duplication of one particular gene endows cells using more flexible regulation strategy to regulate Hh pathway. For example, Ci has three orthologs in vertebrate: Gli1, Gli2, and Gli3 (Bai et al., 2004; Motoyama et al., 2003). Gli1 and Gli2 act primarily as activators, and Gli3 acts both as activator and repressor.

In addition to gene duplication events, the structure of some components in Hh pathway also changed during evolution and therefore the regulation of those components may also change accordingly. For example, significant divergence exists in Smo C-terminal tails across species (Hooper and Scott, 2005; Huangfu and Anderson, 2006; Ingham and McMahon, 2001). In *Drosophila*, the Smo-C tail is longer than the mammalian Smo-C tail. Interestingly, several small molecules bind to vertebrate Smo and act as either agonist or antagonists of Smo (Chen et al., 2002b); however, these molecules have no effect on *Drosophila* Smo (Chen et al., 2002a; Chen et al., 2001; Taipale et al., 2000). These data indicate that the structural differences of Smo between vertebrate and *Drosophila* may result in different regulation of Smo activity.

More dramatic functional divergence has been observed between *Drosophila* and vertebrates in several key components in Hh signaling pathway. In *Drosophila*, Cos2 plays a critical role in Hh signal transduction (Hooper and Scott, 2005; Lum and Beachy, 2004). As a negative regulator, it tethers Ci in the cytoplasm and promotes Ci processing (Wang et al., 2000; Wang and Holmgren, 1999). As a positive regulator, Cos2 is necessary for high threshold downstream gene expression in response to Hh (Wang et al., 2000; Wang and Holmgren, 1999). In zebrafish, morpholino knockdown experiments have indicated that a Cos2 like protein Kif7 acts like a negative regulator of Hh signaling

(Tay et al., 2005). By contrast, RNAi knockdown of two mammalian Cos2-like proteins, Kif7 and Kif27, which share considerable sequence homology with *Drosophila* Cos2, did not significiantly affect Shh signaling in mouse cells (Varjosalo et al., 2006). Similarly, Fu play a critical role in *Drosophila* (Jia and Jiang, 2006; Lum and Beachy, 2004), but its ortholog Stk36 knockout mice have no apparent defects in Hh pathway (Chen et al., 2005; Merchant et al., 2005). As we mentioned before, *Sufu* mutant flies are viable and have no apparent phenotype (Pham et al., 1995; Preat, 1992; Preat et al., 1993), unless *fu* or other Hh pathway component is also mutated (Ohlmeyer and Kalderon, 1998; Wang et al., 2000). Surprisingly, *Sufu* null mouse embryos exhibited very dramatic Hh gain-of-function phenotypes, similar to *ptc1* mutant embryos (Cooper et al., 2005), suggesting that Sufu is a major suppressor of vertebrate Hh pathway. All these data suggest that, although the functions of the most upstream receptor like protein and the most downstream effectors Gli/Ci family proteins are quite conserved, differences in function and regulation do exist for the intermedial signal transducers of Hh pathway.

Recent studies in vertebrate system identified many new components that regulate Hh signal pathway. For example, several components of intraflagellar transport (IFT) machinery that is required for cilia assembly and maintenance, such as Ift172/Wimple, Polaris/Ift88/Ttc10, Ngd5/Ift52, and Kif3a are required for mammalian Hh signalling (Huangfu and Anderson, 2006). A Rab family GTPase Rab23 (Eggenschwiler et al., 2006; Eggenschwiler et al., 2001), a FK506-binding protein family member Fkbp8 (Bulgakov et al., 2004), and a novel protein Sil (Aplan et al., 1990) are all involved in vertebrate Hh signal transduction (Izraeli et al., 1999). However, most of these components seem to be vertebrate specific. Either their *Drosophila* orthologs have no obvious Hh related

functions or there are no indentifiable orthologs in *Drosophila*. It is possible that vertebrates adopt additional new mechanisms to modulate Hh pathway. However, we cannot exclude the possibility that some of these new components also have similar but minor functions which are hard to reveal by their mutations in *Drosophila*.

## Comparison between Hedgehog and Wnt signaling pathways

Wnt family proteins regulate many development processes in both vertebrates and invertebrates (Cadigan and Nusse, 1997; Huelsken and Birchmeier, 2001; Moon et al., 2002; Reya and Clevers, 2005; Wodarz and Nusse, 1998). Three distinct Wnt pathways have been revealed (Kuhl et al., 2000; Peifer and McEwen, 2002). The canonical Wnt pathway stabilizes of β-catenin and determines cell fates. The noncanonical Wnt pathway (Wnt-PCP) affects planar polarity (Moon et al., 2002). The Wnt/Calcium pathway stimulates intracellular Calcium release in a G-protein dependent manner (Kuhl et al., 2000).

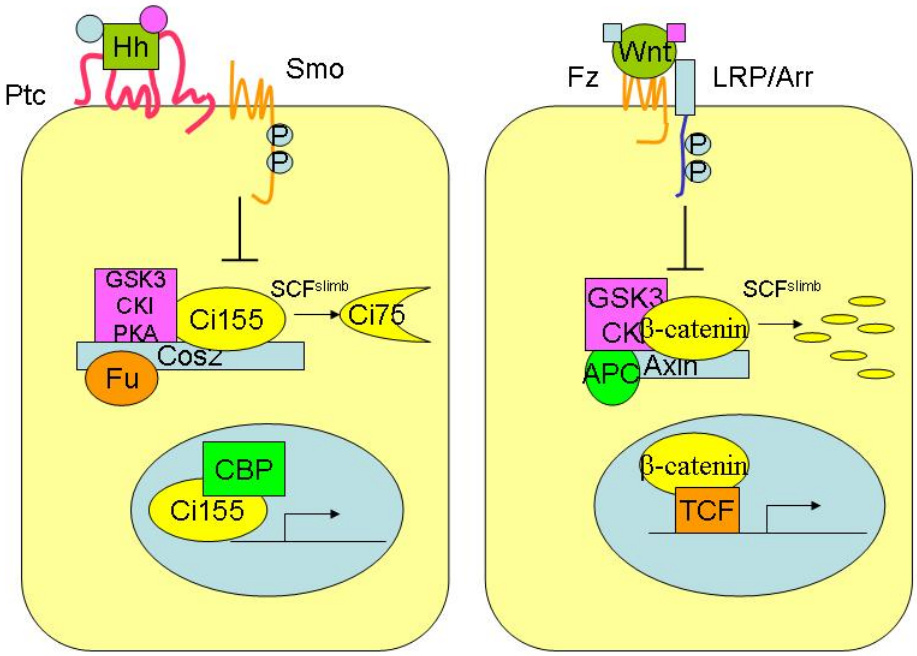



Fig. 1.3- The similarities between Hh pathway (Left) and canonical Wnt pathway (Right). Both Hh and Wnt are secreted proteins and both proteins are modified by lipids. Both Smo and Fz are GPCR like proteins. In response to Hh, Smo is phosphorylated. Correspondingly, the co-receptor of Fz, LRP/Arr is also phosphorylated in response to Wnt. The transcription factors of both pathways (Ci155 and β-catenin) undergo $SCF^{slimb}$ mediated processing in absence of ligand. Big protein complexes and common kinases such as GSK and CKI were involved in phosphorylation and processing of the transcription factors

Several findings have suggested that there are interesting similarities between Wnt and Hh signaling pathways (Jia and Jiang, 2006; Kalderon, 2002; Lum and Beachy, 2004; Nusse, 2003) (Fig. 1.3). Both Wnt and Hh are secreted proteins and both proteins are modified by lipids (Mann and Beachy, 2000). In both pathways, signal transduction is activated by a GPCR like protein. In Wnt pathway, it is Fz. In Hh pathway, it is Smo. In

addition, the key effectors of both pathway (Ci and β-catenin) are phosphorylated by common kinases, GRK3 and CKI in the absence of upstream signals (Amit et al., 2002; Jia et al., 2002; Liu et al., 2002; Price and Kalderon, 2002; Yanagawa et al., 2002). The phosphorylation then results in Slimb/β-TRCP mediated ubiquitination and proteasome based processing (Jiang and Struhl, 1998; Winston et al., 1999). Interestingly, phosphorylation of both Ci and β-catenin is regulated by a large protein complex. In Wnt pathway, a scaffold protein Axin forms a complex with CKIα, GSK3, adenomatous polyposis coli protein (APC), and β-catenin in the absence of Wnt. The whole complex triggers β-catenin phosphorylation and degradation (He et al., 2004; Winston et al., 1999). Similarly, Cos2 interacts with PKA, CKI, GSK3 and Ci in the absence of Hh to regulate Ci phosphorylation and processing (Zhang et al., 2005).

Since Wnt and Hh pathways share so many similarities, studies on one pathway may provide important clues to understand the other pathway. For example, it has been shown that Fz forms multimers (Kaykas et al., 2004). Since Smo is structurally similar to Fz, it will be interesting to determine Smo also forms multimers in order to function.

## The aim of this study

To understand the regulatory mechanism of Smo, we asked three questions: 1) how does Smo transduce the Hh signal to downstream components? 2) What is the kinase that phosphorylates Smo in response to Hh? 3) What is the role of Smo phosphorylation?

In chapter two, the interactions between Smo C-terminal tail and downstream complexes were tested, which provided some clues about how Smo transduces the Hh signal into cells. In chapter three, we provided evidence that PKA and CKI phosphorylate Smo at multiple sites essential for Hh signaling. In chapter four, I identified several cis-

elements that negatively regulate Smo activity. I provided evidence that phosphorylation antagonize those negative effects and activate Smo. In chapter five, we found that Gprk2 positively regulates Hh signaling pathway. We also provided evidence that CKI phosphorylates a specific cluster in Smo C-tail and this phosphorylation appears to be required for Smo endocytosis and Hh signaling activity.

## CHAPTER TWO

### Smoothened transduces Hh signal by physically interacting with Cos2-Fu complex through its C-terminal tail.

#### Introduction

The activation of Hh pathway is mediated by Smo; however, how Smo transduces the Hh signal to downstream components is still unknown. To determine how Smo transduces the Hh signal, a pilot study carried in our lab tested which part of Smo is required for its signaling activity.

We found that GFPSmo overexpression in wing imaginal discs ectopicly induced *dpp* expression in the anterior compartment cells far away from the A/P boundary. However, Smo lacking its C-terminal tail (GFPSmoΔC) failed to induce ectopic *dpp* expression. Further study also showed that SmoΔC failed to rescue *ptc* expression in *smo* mutant clones near the A/P boundary. These results demonstrate that the C-terminal tail of Smo is essential for its signaling activity (Jia et al., 2003).

Furthermore, we found that the membrane tethered forms of Smo C-tail possess constitutive Hh signaling activity. Two membrane tethered forms of SmoC-tail, a Sevenless-Smo chimera protein that contains the extracellular and transmembrane domain of Sevenless and MyrSmoCT that includes a myristoylation signal at the N-terminal of SmoC-tail were made and tested by overexpression in wing imaginal discs. A-compartment cells with either Sev-SmoCT or MyrSmoCT overexpression accumulate

high levels of Ci155 and activated *dpp* expression. By contrast, the soluble form of SmoC-tail did not ectopicly activate Hh signaling, indicating that membrane tethering is essential for Smo activity. Further study showed that MyrSmoCT induced Hh signaling is independent of endogenous Smo and cannot be suppressed by Ptc, indicating the membrane tethered Smo has constitutive activity. However, MyrSmoCT overexpression does not activate *ptc* and *en* expression in most of A-compartment cells, indicating MyrSmoCT is not sufficient to fully activate Hh pathway. To further define the region within SmoCT, a series of deletion mutants were tested in vivo. The results indicate that the C-terminal half of SmoC-tail (730-1035) is critical for Smo activity (Jia et al., 2003).

The above observations suggest that SmoC-tail is important for Hh signaling; however, the mechanism of how Smo communicates with intracellular signaling components remains elusive. Smo is a GPCR like protein, but no evidence has been obtained to support the involvement of G-proteins in physiological Hh signaling process (Hooper and Scott, 2005; Lum and Beachy, 2004). Interestingly, MyrSmoCT overexpression inhibits *ptc* and *en* expression at the A/P boundary and increasing the amount of full-length Smo can reverse this negative effect, suggesting that MyrSmoCT may compete with endogenous Smo for binding to limit amount of downstream components. Since the most proximal downstream components are Fu-Cos2 complexes and extensive genetic screens have failed to identify any Hh signaling components to link Smo and Fu-Cos2 complex together, we highly suspected that Smo may transduce Hh signal by physically interacting with the Fu-Cos2 complex.

Here we provide evidence that Smo interacts with Cos2-Fu complex through its C-terminal tail and the interaction is essential for Hh signaling activity.

## Results

Smo interacts with Cos2-Fu complex through its C-terminal tail.

To determine whether there is physical interaction between Smo and Cos2-Fu complex, I transfected *Drosophila* S2 cells with either Myc tagged wild type Smo (Myc-Smo) or C tail deletion form (Myc-SmoΔC) together with HA tagged Cos2(HA-Cos2), Fu and Ci. Cell extracts were immunoprecipitated with a mouse anti-Myc antibody and the immunoprecipitate products were analyzed by Western blot. Myc-Smo but not Myc-SmoΔC pulled down Cos2 and Fu (Fig. 2.1A), indicating that Smo C-tail is essential for the interaction between Smo and Cos2-Fu complex. Ci cannot be constitutively pulled down in this assay, suggesting that the interaction between Ci and Smo is dynamic. It is possible that once Smo bind to Cos2-Fu-Ci complex, Ci is released from the whole complex. I also found that overexpressed Myc-Smo also pulled down endogenous Cos2 and Fu in S2 cells (Fig. 2.1B). Since there is no endogenous Ci in S2 cells, the interaction between Smo and Cos2-Fu complex is independent of Ci. Interestingly, Cos2 overexpression *per se* does not increase the amount of Cos2 bound to Smo (Fig. 2.1C), indicating that the endogenous Cos2 is enough to saturate the binding to Smo. However, Fu overexpression increases the amount of Fu pulled down by Smo. Furthermore, Fu and Cos2 coexpression also increase the amount of Cos2 bound to Smo (Fig. 2.1C). These data suggests that Fu-Smo interaction is independent of Cos2, while Cos2-Smo interaction at least partially depends on Fu.

To test whether the interaction between Smo and Cos-Fu complex also happens *in vivo*, Myc-Smo or Myc-SmoΔC was expressed in *Drosophila* wing imaginal discs using the *MS1096* Gal4 driver. Wing discs extract was immunoprecipitated with mouse

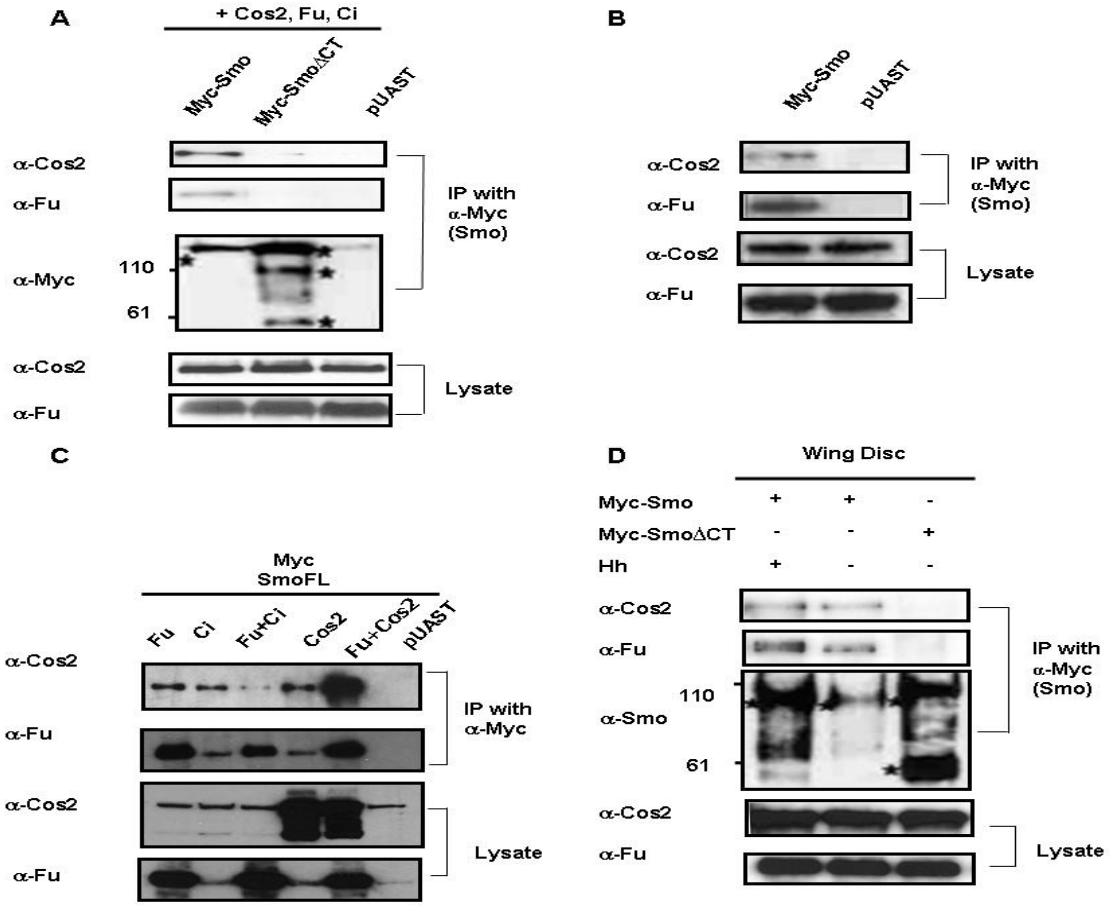



Fig. 2.1- The physical interactions between SmoC-tail and Cos2-Fu complex. S2 cells were transfected with Myc-Smo or Myc-SmoΔCT expressing contruct with (A) or without (B) Cos2, Fu, and Ci expressing constructs. The blank expressing vector pUAST was used as a control. Cell extracts were immunoprecipitated with mouse anti-Myc antibody. Immunoprecipitates and 5% whole cell lysate were assayed by western blot with indicated antibodies. Myc-Smo but not Myc-SmoΔCT pulled down overexpressed as well as endogenous Cos2 and Fu. Overexpressed Myc-Smo and Myc-SmoΔCT exhibit as slow migrating bands (indicated by asterisks) as reported before (Stone et al., 1996).
(C) S2 cells were transfected with Myc-Smo expressing construct in conjugation with Fu, Cos2 and Ci expressing constructs in different combinations as indicated. Cell extracts were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with indicated antibodies. Overexpressing Cos2 does not result in more Cos2 pulled down by Smo. However, overexpressing Fu leads to more Fu pulled down by Smo. Coexpressing Fu with Cos2 results in more Cos2 pulled down by Smo. (D) Cell extracts were prepared from 400 wing discs expressing UAS-Myc-Smo or UAS-Myc-SmoΔCT in conjugation with *UAS-Hh* under control of *MS1096.* Wing disc extracts were immunoprecipted with anti-Myc antibody, followed by immunoblotting with indicated antibody. Hh stimnulation stabilizes Smo (indicated by asterisks) and results in a slight increase of Cos2 and Fu pulled down by Smo.

anti-Myc antibody, followed by westernblotting with anti-Cos2, anti-Fu, and anti-Ci antibodies. Myc-Smo but not Myc-SmoΔC pulled down Cos2 and Fu (Fig. 2.1D). Ci was not detectable from the immunoprecipitate products. To determine whether Hh regulates the interaction between Smo and Cos2-Fu complex, we coexpressed *UAS-hh* together with *UAS-Myc-Smo* in wing imaginal discs by using Gal4 driver *MS1096*. As shown in Fig.2.1D, Hh expression stabilized Smo. But there was only a slight increase of the amount of Cos2 and Fu pulled down by Myc-Smo, which is likely due to the increased levels of Smo in response to Hh. Interestingly, a significant amount of Fu bound to Smo is phosphorylated in the Hh expressing discs, as indicated by its mobility shift (Fig.2.1D).

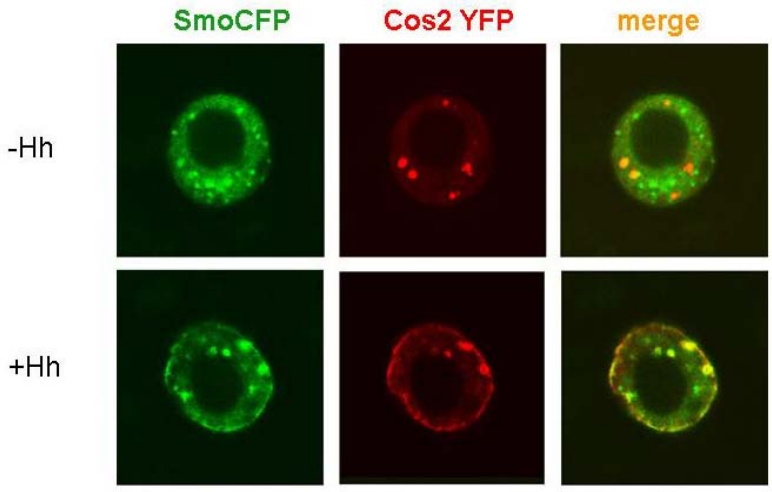



Fig. 2.2- The subcellular localization of Smo-Cos2 Complex is changed in response to Hh. S2 cells were transfected with SmoCFP and Cos2YFP expressing constructs, followed by incubating with Hh conditional medium (lower panel) or control medium (upper panel). Cells were fixed and observed by confocol microscope. Green channel indicates the CFP signals and red channel indicates the YFP signal. Cos2 and Smo is colocalized no matter there is Hh or no. However, the majority of Smo-Cos2 complex moved to cell surface in response to Hh.

Since immunoprecipitation cannot distinguish the subcellular localization of the complex, I transfected S2 cells with CFP tagged Smo (SmoCFP) and YFP tagged Cos2 (Cos2YFP) and stimulated cells with or without Hh. As showed in Fig. 2.2, no matter there was Hh or not, the majority of SmoCFP was colocalized with Cos2YFP. However, with Hh stimulation, most of the Smo-Cos2 complex moved to the cell surface. These data suggest that Hh may not change the affinity between Smo and Cos2-Fu complex but change the subcellular localization of the complex, which may re-localize the whole complex to a subcellular compartment favorable for Hh signal transduction.

The interaction between Smo and Cos2-Fu complex is essential for Hh signaling.

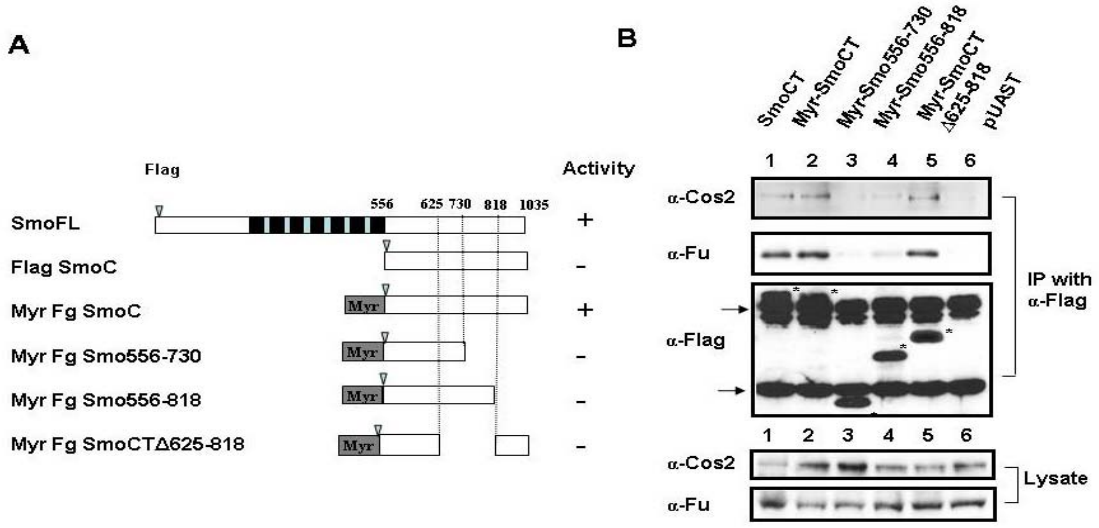



Fig. 2.3- The interaction between Smo and Cos2 is essential for Hh signaling. (A) Fg-tagged Smo truncation mutants. Smo transmembrane domains were indicated by the black boxes. Gray boxs and triangles indicate myristoylation signal and Flag tags, respectively. The amino acid residues that demarcate each deletion mutant are indicated. Individual constructs were assayed for Hh signaling activity by overexpression using UAS/Gal4 system in wing discs. Activity is scored when full-length Ci was stabilized and *dpp-lacZ* was activated in anterior cells away from A/P boundary. (B) S2 cells were transfected with various Smo deletion mutants as indicated. Cells extracts were immnoprecipated by anti-Fg antibody, followed by immunoblotting with antibodies as indicated. The Smo truncations were indicated by asterisks. Arrows indicate the heavy chain and light chain of IgG.

To determine Cos2-Smo interaction domain, a series of membrane tethered forms of Smo truncation mutants (Fig. 2.3A) were transfected to S2 cells. Cell extracts were immunoprecipitated by mouse anti-Fg antibody, followed by Western blot analysis with anti-Cos2 and anti-Fu antibody. As showed in Fig. 2.3B, both Myr-SmoCT and Myr-SmoCT730-1035 are able to pull down endogenous Cos2 and Fu, indicating the Smo C-tail is sufficient to mediate the interaction with Cos2-Fu complex. Consistent with their inability to activate Hh signaling, Myr-Smo556-730 and Myr-Smo556-818 bound to diminishing levels of Cos2/Fu. Interestingly, although Mry-SmoΔ625-818 does not have any *in vivo* activity, it still pulled down Cos2/Fu complex at the same level as Mry-SmoCT, suggesting that the interaction between Smo and Cos2/Fu is not sufficient to activate Hh pathway. Consistent with this, the soluble form SmoCT also interacted with Cos2/Fu, although it did not have any *in vivo* activity.

To define the Cos2 domain(s) that mediate Smo binding, a series of HA tagged Cos2 truncation mutants were generated (Fig. 2.4A) and transfected to S2 cells in conjugation with Myc-Smo. The ability of Cos2 truncation mutants to bind to Smo was examined by immunoprecipitation with anti-Myc antibody. As showed in Fig. 2.4B, as least two domains in Cos2 interact with Smo: the microtubule binding domain and the C-tail cargo domain. Both HA-Cos2MB, HA-Cos2CT1 and HA-Cos2CT2 were pulled down by Myc-Smo robustly. HA-CosΔN2 also strongly binds to Smo. In contrast, HA-Cos2CC was not pulled down by Myc-Smo. Interestingly, Myc-Smo pulled down relatively less HA-Cos2, HA-Cos2ΔN1, and HA-Cos2ΔC. One possibility is some interaction domains are masked in this large protein and the binding with Fu may change Cos2 conformation and help Cos2-Smo interaction. Intriguingly, both HA-Cos2ΔN2 and

HA-Cos2C1 but not HA-Cos2CC overexpression in wing imaginal discs blocked Hh signaling activity (Jia et al., 2003), indicating those Cos2 truncations containing Smo binding domains may titrate out endogenous Smo and prevent Smo interacting with endogenous Cos/Fu complex and therefore inhibit Hh signaling activity. Consistently, Cos2ΔN2 and GFP-Smo coexpression restored Hh signaling activity (Jia et al., 2003). All these data suggest that the interaction between Smo and Cos2-Fu complex is essential for Hh signaling.

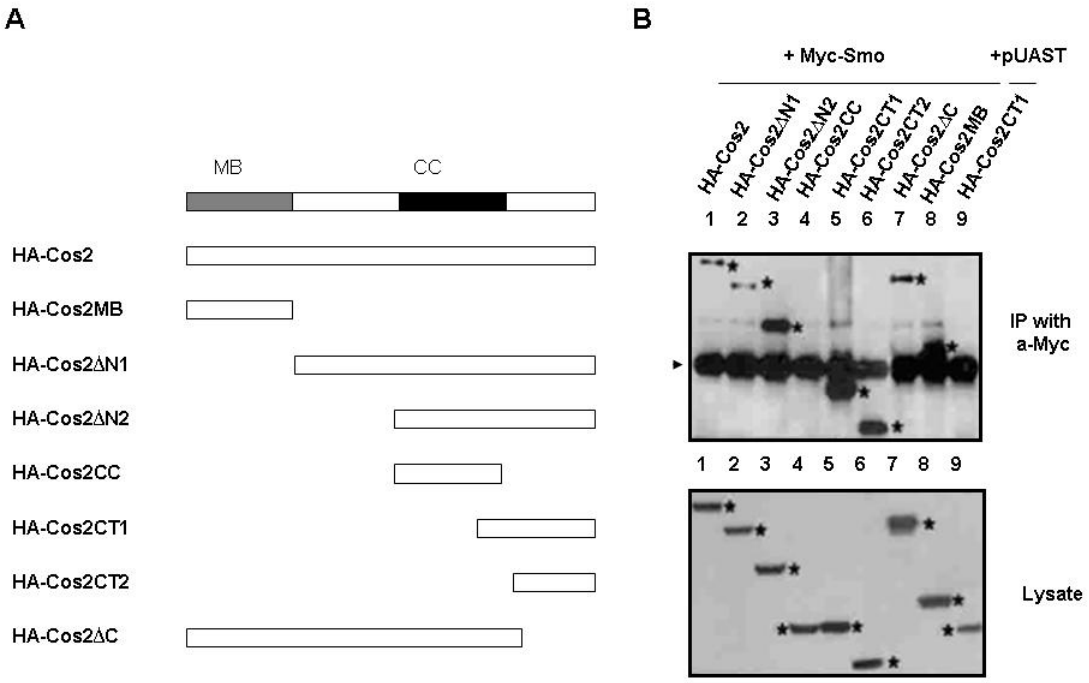



Fig. 2.4- The Smo interaction domains in Cos2. (A) HA-tagged Cos2 truncation mutants. The gray and black boxes indicate the microtubule binding domain (MB) and coil-coil (CC) domain. (B) S2 cells were transfected with Myc-Smo and various HA tagged Cos2 truncation mutants. Cell extracts were immunoprecipitated by anti-Myc antibody, followed by immunoblotting with anti-HA antibody. 5% total cell lysates were also subjected to western blotting with anti-HA antodody. Asterisks indicate the position of HA tagged Cos2 proteins expressed from corresponding constructs. Arrow head indicates IgG.

## Discussion

The seven transmembrane protein Smo plays central roles in Hh signal transduction (Ingham and McMahon, 2001; Jia and Jiang, 2006; Lum and Beachy, 2004). In this study, I provide biochemical evidence that Smo interacts with Cos2/Fu complex through its C-terminal tail. I also mapped the interaction domain between Cos2 and Smo. In addition, our data suggest that the interaction between Smo and Cos2-Fu complex is essential for Hh signaling. These findings filled the gap between Smo and downstream components, indicating that Smo transduces Hh signal by directly recruiting the Cos2/Fu complex to membrane rather than by activating a signaling cascade involving G proteins.

How does the Smo-Cos2-Fu interaction transmit Hh signal?

At the time we published our findings, several other groups also reported that Smo transduces Hh signal by interacting with Cos2-Fu complex directly (Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). Although demonstrating the interaction between Smo and the Cos2-Fu complex links Hh signaling generator and effector together, these studies do not provide a simple explanation as to how the interaction between these proteins transmits the Hh signal.

As we know that different levels of Hh induce different cellular responses (Alexandre et al., 1996; Strigini and Cohen, 1997). Low levels of Hh are enough to block Ci processing and induce *dpp* expression. Medium levels of Hh begin to activate *ptc* expression and high levels of Hh are required to activate *en* expression. In this study, we found that there are constitutive interactions between Smo and Cos2. The binding affinity between these two proteins does not seem to change in response to Hh. A similar finding was also reported by Lum *et al.* (Lum et al., 2003b). However, since levels of Smo

increase dramatically with the stimulation of Hh (Denef et al., 2000), the pool of Smo-Cos2 complex should also increase proportionally, which may contribute to high threshold Hh responses. However, in stead of quantative differences, the interaction between Smo and Cos2 may have some qualitative difference such as change of interaction domains or protein conformation in response to different levels of Hh. In addition, since Smo translocates to cell surface in response to Hh, Cos2-Fu complex is also relocalized with Smo. The change of subcellular localization may also change Cos2 and Fu activity correspondingly.

In response to Hh, Ci processing is blocked (Aza-Blanc et al., 1997). It has been reported that Cos2 is essential for processing and cytoplasmic tethering of Ci (Wang et al., 2000; Wang and Holmgren, 2000; Zhang et al., 2005). It is possible that Smo-Cos2 interaction, especially when there is Hh, interferes with Cos2's inhibitory effects on Ci. In this study we found that Smo interacts with both the N and C terminal parts of Cos2. Interestingly, these two domains are also essential for Cos2 to interact with PKA, CKI, and GSK (Zhang et al., 2005). Since recruitment of kinases by Cos2 is necessary for Ci phosphorylation and processing, it is likely that the interactions between Cos2 and kinases are interrupted by Cos2-Smo interaction. Strikingly, Cos2 dissociated from PKA, CKI, and GSK3 in the presence of Myr-SmoCT (Zhang et al., 2005) and Myr-SmoCT overexpression could block Ci processing. A paralleled study carried out by Ruel ***et al.*** (Ruel et al., 2003) found that Smo pulled down Cos2, Fu and Ci in the absence of Hh. However, with the stimulation of Hh, Ci was largely absence from Smo-Cos2 complex. These finding provided another explanation of how Hh prevents Ci processing. However, in our study, we barely detected any Ci in Smo-Cos2 complex at any conditions. While,

Lum *et al.* (Lum et al., 2003b) showed that Ci was constitutively detectable in Smo-Cos2 complex regardless to the Hh stimulation. These inconsistent data suggested that the interaction between Ci and Smo-Cos2 complex might be very dynamic. The difference between different groups may due to the different wash stringency used during immunoprecipitation.

In this study, we found that Myr-SmoCT overexpression inhibits *ptc* and *en* expression in the wing imaginal discs near the A/P boundary. Since Myr-SmoCT interacts with Cos2 and the activity of MyrSmoCT is not enough to trigger high threshold Hh responses, overexpression of MyrSmoCT may titrate out Cos2 and inhibit the interaction between endogenous Smo and Cos2 and therefore block *ptc* and *en* expression. These data suggest that Smo-Cos2 interaction is important for high threshold Hh responses. However, It is still a mystery how Smo-Cos2 interaction trigger the high threshold Hh responses. It has been showed that Fu activity is required for high threshold Hh responses (Ohlmeyer and Kalderon, 1998; Sanchez-Herrero et al., 1996). In response to Hh, Fu is phosphorylated and activated (Therond et al., 1996). Fu activity could counteract with the negative effects of Sufu (Ohlmeyer and Kalderon, 1998), likely due to direct phosphorylation (Ho et al., 2005; Lum et al., 2003b), and then activate the expression of high threshold Hh target gene such as *ptc* and *en*. In response to high levels of Hh, it is possible that Smo and Cos-Fu complex interaction somehow activates Fu and therefore suppresses Sufu and activate high threshold Hh responses. Although Myr-SmoCT interacts with Cos2-Fu complex, it is possible that it could not activate Fu and therefore is not sufficient to transduce high threshold Hh signaling. Consistent with this hypothesis, Myr-SmoCT overexpression could activate both *ptc* and *en* expression in the

*Sufu* mutant clones (Jia et al., 2003). It is still elusive how Fu activity is regulated. It is also not clear whether phosphorylation of Fu plays any role in Fu activation.

Interestingly, Hooper's group found that SmoC-tail is sufficient to induce a full spectrum of Hh responses when it is fused to the extracellular and transmembrane domain of Fz, another seven transmembrane protein functioning in Wnt pathway (Hooper, 2003). These data suggest that SmoC-tail contains all the necessary components to activate Hh signaling pathway, but it has to be properly regulated. It has been shown that the N-terminal part of Fz could be dimmerized and which is essential for Fz function (Kaykas et al., 2004). It is possible that dimmerization of Smo is also play pivotal role in Hh signal transduction. Although there is no direct evidence for Smo dimmerization, several lines of evidence all point to that direction (Hooper, 2003). The interaction between Smo and Cos2-Fu complex and Smo dimmerization may provide a good platform to facilitate Fu phosphorylation and activation. All these possibilities need to be tested in future.

The dual roles of Cos2 in Hh signaling pathway

Cos2 was first identified as a negative regulator that is essential for Ci processing and cytoplasmic tethering (Capdevila and Guerrero, 1994; Grau and Simpson, 1987). In addition to those negative roles in Hh pathway, the recent studies demonstrated that Cos2 also plays a positive role in Hh pathway (Wang et al., 2000; Wang and Holmgren, 2000). In addition to blocking Hh signal transduction in anterior compartment cells away from the compartment boundary, Cos2 is required for transducing high levels of Hh signaling activity by antagonizing Sufu in anterior compartment cells near the A/P boundary (Wang et al., 2000). In the *cos2* mutant clones, *en* expression was blocked, while, the

inhibition of Sufu at the same time restored *en* expression (Wang et al., 2000). Interestingly, Lum *et al.* found that Cos2 is required for Fu stabilization and Fu dependent Sufu phosphorylation (Lum et al., 2003b), which provided an explanation for Cos2-mediated inactivation of Sufu.

Here, we found that Cos2 interacts with Smo and the interaction is essential for Hh signal transduction. The requirement of Cos2 for highest levels of Hh signaling transduction may reflect the essential role of Cos2-Smo interactions (Lum et al., 2003b). Interestingly, the activity of Cos2 appears to depend on the levels of Smo, the high levels of Smo in maximally stimulated cells can convert Cos2 from a negative regulator to a positive regulator. In the absence of Hh, Smo level is low and therefore Cos2 mainly play negative roles and keep cells in a unstimulate state. In response to low levels of Hh, Smo level is increased and the negative effects of Cos2 are blocked. However, the Smo activity may be still not enough to stimulate the positive roles of Cos2. In response to high levels of Hh, Smo levels increase more dramatically and the activated Smo converts Cos2 to a positive regulator which may promote Fu activation and activate high threshold Hh responses. However, increasing Smo level alone is not enough to fully activate the Hh pathway (Hooper, 2003; Jia et al., 2003). The stimulation of Smo by Hh is necessary for Cos2 to play its positive roles. It is still unclear how Cos2 functions as a positive regulator. Changing conformation or recruiting other molecules by binding to highly active form of Smo (probably Smo dimmer) may facilitate Fu activation and therefore activate high threshold Hh responses.

<u>The similarity between Wnt and Hh signaling pathway</u>

As introduced before, Wnt and Hh pathways are like siblings and keep use similar strategy to transmit signals. Both Fz and Smo are GPCR like seven transmembrane proteins. Interestingly, the Fz-SmoCT chimera protein which includes the extracellular and transmembrane domain of Fz and the C-terminal tail of Smo could stimulate the full spectrum of Hh signaling responces in response to different levels of Wnt (Hooper, 2003). These data suggested that Fz and Smo may share similar regulating strategies to interpret different levels of ligand. For example, they may form dimmer or multimmer to transduce high threshold signaling activaties.

As signal transducers, it was unclear how Fz and Smo transduce signals to cytoplasmic components until recently. Several studies have shown that these transmembrane proteins directly interacted with their cognate cytosolic components. In the Wnt/Calcium pathway, it has been shown that G protein is involved in transmitting signal to downstream (Kuhl et al., 2000); however, there is little evidence shown that Fz-β-cateinin or Fz-PCP pathway was disrupted or phenocopied by manipulating G-protein activity. Similar observations also obtained in Hh signaling pathway (Kalderon, 2002). Knocking down different subtypes of G protein by RNAi does not perturb Hh signaling responses in *Drosophila* cultured cells (Lum et al., 2003b). Other studies also suggested if any G-protein is involved in Hh signal transduction, its function may not be direct. All these studies point to one direction that Fz or Smo may interact with the downstream components directly to transduce upstream signal.

In this study, we found that Smo interact directly with downstream Cos2-Fu complex to transduce Hh signal. Interestingly, another recent study in mammalian cells showed that Fz interacts with the PDZ domain of Disheveled (Dvl) directly and this

interaction is essential for canonic Wnt signal transduction (Wong et al., 2003). Dvl is a protein including DIX, PDZ, and DEP domains and it is required for both β-cateinin and PCP pathway (Krasnow et al., 1995; Noordermeer et al., 1994; Shulman et al., 1998; Siegfried et al., 1994). The genetics analysis put Dvl downstream of Fz and upstream of other Wnt pathway components (Boutros and Mlodzik, 1999). β-cateinin pathways signaling accompanied by recruiting Dvl to the cell membrane (Boutros and Mlodzik, 1999; Yanagawa et al., 1995), while, the asymmetry distribution of Dvl and Fz provides the direction cues to PCP pathway (Adler, 2002; Peifer and McEwen, 2002). Although Cos2 and Dsh interact with the membrane signal transducer directly, there is no obvious similarity between the functions of these two proteins. Since Smo has a considerable larger C-terminal tail comparing to Fz, it is conceivable that Dvl may mimic the function of Smo C-tail (Kalderon, 2002). Strikingly, in Wnt- β-cateinin pathway, LRP5/6 (the *Drosophila* homolog named Arrow), the co-receptor of Wnt, interacts with another cytosolic component of Wnt pathway: Axin (Mao et al., 2001). Just like Cos2, Axin is a large scaffold like protein that recruits APC and kinases to phosphorylate β-cateinin and promotes β-cateinin degradation (He et al., 2004; Winston et al., 1999). As mentioned before, Smo-Cos2 interaction inhibits the negative role of Cos2 and block Ci-processing. Quite similarly, the interaction between LRP5/6 and Axin interferes with its function in β-cateinin degradation. Importantly, LRP5/6-Axin interaction inhibits β-cateinin degradation not only by promoting Axin degradation but also by using other unknown mechanism (Mao et al., 2001). Interestingly, Ruel *et al*. (Ruel et al., 2003) also found that *hh* or *smo* overexpression in anterior compartment cells reduces Cos2 and Fu levels. In addition, our group's later studies showed the interaction between Smo and Cos2

complex disrupts the interaction between kinases and Cos2 (Zhang et al., 2005). It will be interesting to test whether the interaction between LRP5/6 and Axin also could disrupt the interaction between GSK3 and Axin. Furthermore, in our study, we found Cos2 plays a positive role in Hh signaling pathway. It is also worth to test whether Axin also has dual roles in Wnt signaling transduction.

## Materials and Methods

Plasmid construction and transgenes

To construct UAS-MycSmo, six copies of Myc tag sequences were amplified by PCR and inserted into the SfiI sites (amino acid 35) of Smo cDNA. The resulting fusion gene was subcloned into a pUAST vector between the NotI and XhoI sites. MycSmoΔC was derived from MycSmo by introducing a stop codon at amino acid 555. To construct UAS-FgSmoCT, *smo* cDNA encoding the C-terminal region of Smo from amino acid 556 to 1035 was amplified by PCR and inserted between the BglII and XhoI sites of a pUASTFlag vector. To generate UAS-Myr-SmoCT, a myristoylation signal from Src (MGNKCCSKRQ) was inserted at the N-terminus of FgSmoCT. Membrane-tethered deletion mutants of SmoCT were generated by a PCR-based approach using appropriate primers. HA-Cos2 contained two copies of HA tags at the N terminus of Cos2 (Wang et al. 2000). HACos2ΔC was derived from HA-Cos2 by introducing a stop codon after amino acid 993. HA-Cos2ΔN1, HA-Cos2N2, HA-Cos2CT1, and HACos2CT2 contain two copies of HA tag fused N-terminal to amino acids 389, 642, 906, and 991, respectively. HA-Cos2CC was derived from HA-Cos2ΔN2 by introducing a stop codon after amino acid 993.

Cell culture and transfection

S2 cells were cultured in Schneider's Drosophila Medium (Invitrogen) with 10% fetal bovine serum (Sigma). Routinely, S2 cells are grown at 1-5 × $10^7$ cells/ml and are split into fresh medium at 1:5 dilution every 3 days.

To make Hh-conditioned medium, 4 × $10^7$ Hh-N producing cells (Lum et al., 2003a) were seeded in 10 ml fresh S2 cell culture medium with 0.7mM $CuSO_4$. Cells were

incubate in 25 ºC incubator for 24 hours and then the cell suspension was transferred into a sterile tube before centrifuging at 1000 × g for 5 minutes. The Hh-conditioned medium (supernatant) was transferred into a new tube and store at 4 ºC. The Hh-conditioned medium could be stored for up to 1 week before losing activity.

S2 cell transfection was carried out by using standard calcium phosphate transfection procedure. Briefly, $1\times 10^7$ S2 cells were seeded on 10cm dishes in 10 ml fresh culture medium one day before transfection. An *ub-Gal4* construct was cotransfected with pUAST expression vectors for all the transfection. 4 μg DNA for *ub-Gal4* and 2 μg DNA for each pUAST expression constructs were used in a typical transfection experiment and pcDNA was added to make the final amount of DNA to 20 μg. Solution A (20 μg DNA, 60ul 2M $CaCl_2$, adding water to 500ul) and solution B (500ul 2× HBS (50 mM HEPES, 1.5 mM $Na_2HPO_4$, 280 mM NaCl, pH 7.1)) were mixed by continue bubbling the mixture. The resulting solution was incubated at room temperature for 30 minuets followed by adding to the cells slowly with swirling the plate to mix the solution and medium well. Transfected cells were incubated in 25 ºC incubator for 24 hours, followed by changing medium and incubating for another 16-24 hours in 25 ºC incubator before harvest.

Immunoprecipitation and western blot analysis

Cell extracts were prepared by incubating cell pellets with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM $Na_3VO_4$, 1% NP40, 10% Glycerol, 1.5 mM EDTA pH 8.0, protease inhibitor tablets (Roche)) for 30 minuets at 4°C, followed by centrifuging for 10 minuets at 14000g. The resulting supernatant was incubated with proper antibodies (20ng/ml for mouse anti-Myc antibody and 400ng/ml for mouse anti-

Flag antibody) for 1 hour. Then the UltraLink immobilized Protein A (Pierce) beads were added in the lysate and incubated for 2 hours. Then the beads were washed three times by lysis buffer, followed by adding in SDS sample buffer. Western blot analysis was performed by using standard protocols as previously described. The antibodies used were mouse anti-Myc, 9E10 and mouse anti-HA, F7 (Santa Cruz); mouse anti-Flag, M2 (Sigma); mouse anti-Cos2 and rabbit anti-Fu (Ascano et al., 2002); and rat anti-Ci, 2A1 (Motzny and Holmgren, 1995).

## CHAPTER THREE

### Phosphorylation of Smo by PKA and CKI is essential for Smo activation.

#### Introduction

The seven transmembrane protein Smo transduce Hh signal in both invertebrate and vertebrate; however, the mechanism by which Smo is regulated remains largely unknown. In response to Hh, Smo is hyperphosphorylated and accumulated on cell surface (Denef et al., 2000). However, the biological function of Smo phosphorylation is still elusive. In addition, the kinase that is responsible for Hh induced Smo phosphorylation has not been identified yet.

PKA was initially identified as a negative regulator of Hh signaling pathway (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). PKA phosphorylates full-length Ci (Ci155) and promotes its further phosphorylation by GSK3 and CKI (Jia et al., 2002; Jia et al., 2005; Price and Kalderon, 2002). Hyperphosphorylated Ci is targeted for proteolytic processing to generate repressor form of Ci75 which inhibits Hh downstream genes such as *dpp*. Consistent with this, overexpressing a constitutive active form of PKA catalytic subunit mC* blocks the accumulation of Ci155 and Hh target gene expression. Interestingly, we found that modestly expression of mC* stabilized Smo and enhanced Hh pathway activity (Jia et al., 2004). Expressing a weak line of UAS-mC* expressing in wing porch region using *MS1096 Gal4* driver resulted in anterior expansion of both the *ptc* and *en* expression

domains. Since high levels of Hh convert Ci155 to an active unstable form (Ohlmeyer and Kalderon, 1998), the Ci level in cells abutting A/P boundary is low. Consistent with the expansion of *en* expressing domain, the "low Ci155" domain also expanded to more anterior cells. These data suggested that modest increasing PKA activity elevates Hh signaling activity and converts Hh responses from low to high in more anterior cells. Further study indicated that mC* exerts positive influence on Hh signaling in a cell-autonomous manner (Jia et al., 2003).

Conversely, blocking PKA activity by overexpressing a mutant PKA regulator subunit R* with *apGal4*, a dorsal compartment specific Gal4 driver, blocks Hh-induced Smo accumulation and *en* expression in dorsal compartment cells. In addition, in the mitotic clones mutant for *DC0* which encode the major isoform of PKA catalytic subunit (Kalderon and Rubin, 1988), Smo accumulation was attenuated and *en* expression was reduced. These data suggested that PKA activity is essential for Smo accumulation and high threshold Hh responses.

Similar to PKA, CKIα activity has also been implicated in promoting Ci processing and inhibiting Hh pathway activity (Jia et al., 2005; Price and Kalderon, 2002). Surprisingly, we found that expressing a CKIα RNAi construct blocked Smo accumulation and attenuated *ptc* and *en* expression, indicating that CKIα also play dual role in Hh pathway and its activity is required for Smo accumulation and high threshold Hh responses (Jia et al., 2004).

All these data indicates that PKA and CKIα positively regulate Hh pathway upstream of Smo. Since Smo is phosphorylated in response to Hh (Denef et al., 2000), we

highly suspected that PKA and CKIα may phosphorylate Smo directly and regulate Smo activity.

In this study, we provide evidence that PKA and CKI phosphorylate Smo at multiple sites. PKA and CKI phosphorylation deficient forms of Smo fail to accumulate on cell surface and lose signaling activity. In contrast, Smo variants with both PKA and CKI sites converted to Aspartic amino acids exhibit enhanced cell surface expression and constitutive activity. Furthermore, we find the levels of Smo cell surface accumulation and activity correlates with its levels of phosphorylation.

## Results

Direct phosphorylate of Smo by PKA and CKI

Smo C-terminal tail contains five predicted PKA sites including $Ser_{667,}$ $Ser_{687}$, and $Ser_{740}$ that fall into the following consensus sequence: RR/KXS (X refers to any amino acid, and the underlined residues refer to the target site)(Kemp and Pearson, 1990) (Fig. 3.1A). Interestingly, each of these PKA sites is followed by two predicted CKI sites of following consensus $_{(p)}$SXXSXXS ($_{(p)}$S refers to a phospho-serine or phospho-threonine, X refers to any amino acid, and the underlined residues refer to the target site)(Flotow et al., 1990; Flotow and Roach, 1989). In addition, these phosphorylation clusters are conserved in A. gambiae Smo (Fig. 3.1B). To determine whether Smo could be phosphorylated by PKA and CKI directly, I carried out *in vitro* kinase assay. Three GST fusion proteins containing Smo C-tail fragment from aa656 to aa755 were generated: GST-Smo, GST-Smo$^{-PKA123}$ has three PKA sites mutated to Ala, and GST-Smo$^{-CKI}$ has six CKI sites mutated to Ala (Fig. 3.1A). GST-Smo was phosphorylated by PKA as well as CKI after primed phosphorylation by PKA (Fig. 3.1C). Mutating PKA sites in GST-Smo abolished phosphorylation by PKA and CKI. Mutating CKI sites blocked PKA primed phosphorylation by CKI but did not affect phosphorylation by PKA. These results suggest that PKA phosphorylates Smo at Ser667, Ser687, and Ser740, which primes its further phosphorylation by CKI at the downstream sites.

To determine whether PKA and CKI could phosphorylate Smo in vivo, we transfected *Drosophila* S2 cells with Flag tagged wild type Smo (Fg-Smo) or Flag tagged Smo with PKA sites mutated to alanine (Fg-Smo$^{-PKA123}$) together with mC* and/or CKI kinase domain (CKIKD). As showed in Fig. 3.1D, PKA cotransfection induced

electrophoretic mobility shift of Fg-Smo, indicative of Smo phosphorylation. The mobility shift of Fg-Smo was further enhanced by addition of CKI. Moreover, PKA and CKI induced accumulation of Fg-Smo. The tubulin in the cell lysate was blotted as an internal loading control. In contrast, PKA and CKI cause no significant change in either mobility shift or the level of Fg-Smo$^{-PKA123}$. These data indicate that PKA and CKI phosphorylate Smo at those predicted sites, which induce accumulation of Smo.

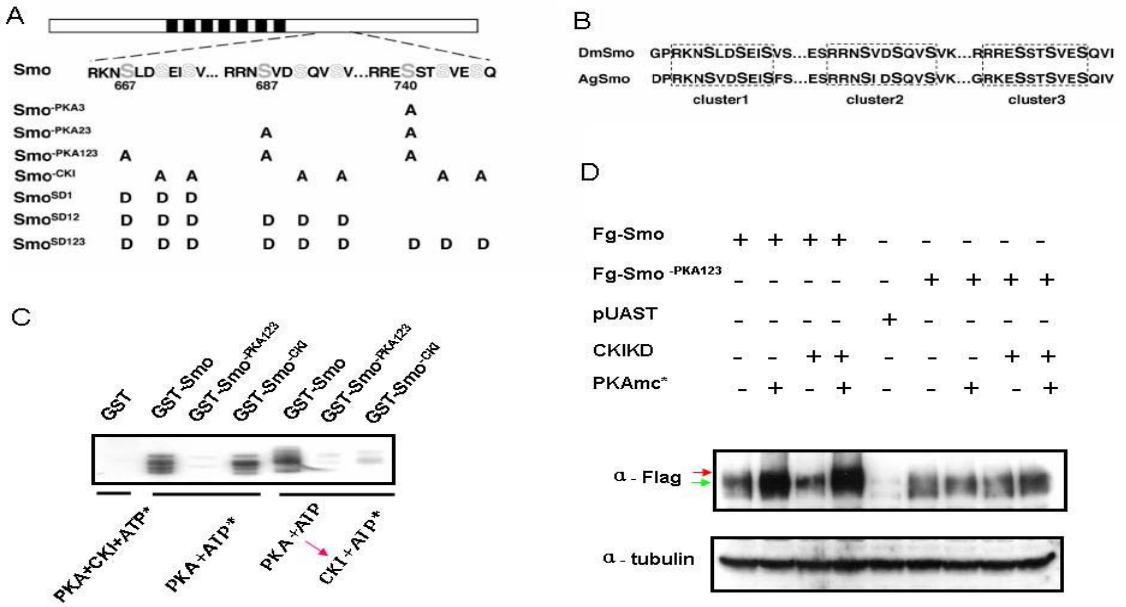



Fig. 3.1- Phosphorylation of Smo by PKA and CKI. (A) Full length Smo with three phosphorylation clusters shown underneath. Large gray and open letters indicate the PKA and CKI sites, respectively. A list of Smo variants with corresponding amino acid substitutions is shown below. (B) Sequence alignment of the three phosphorylation clusters between *Drosophila* Smo (DmSmo) and *Anopheles* Smo (AgSmo). (C) Autoradiograph of an in vitro kinase assay. Equal amount of GST fusion proteins were incubated with recombinant PKA and [γ- $^{32}$P]ATP(ATP*) or incubated with recombinant CKI in presence of ATP* following incubation with PKA and cold ATP(ATP). PKA and CKI sites mutation abolished GST-Smo phosphorylated by PKA and CKI. (D) S2 cells were transfected with Fg-Smo or Fg-Smo$^{-PKA123}$ in conjugation with PKAmc* and CKI kinase domain (CKIKD) in different combinations as indicated. Cell extracts were immunoprecipitated by anti-Fg antibodies, followed by immunoblotting with anti-Flag antibody. The slow mobility of Fg-Smo indicates hyperphosphorylation forms of Smo, showed by the red arrow. 10% total extracts were also analyzed by immunobloting with anti-tubulin antibody to indicate equal extract inputs.

Hh induced Smo phosphorylation is PKA and CKI activity dependent.

To determine whether Hh induced Smo phosphorylation is PKA and CKI activity dependent, S2 cells transfected with Fg-Smo were treated with Hh conditional medium in combine with or without PKA inhibitor H89 (Chijiwa et al., 1990) and CKI inhibitor CKI-7 (Chijiwa et al., 1989). As shown in Fig. 3.2A, Hh induced mobility shift and accumulation of Fg-Smo, which were diminished by H89 treatment and nearly abolished by combined treatment of with H89 and CKI-7. These data suggest that Hh-induced Smo phosphorylation is mediated by PKA and CKI.

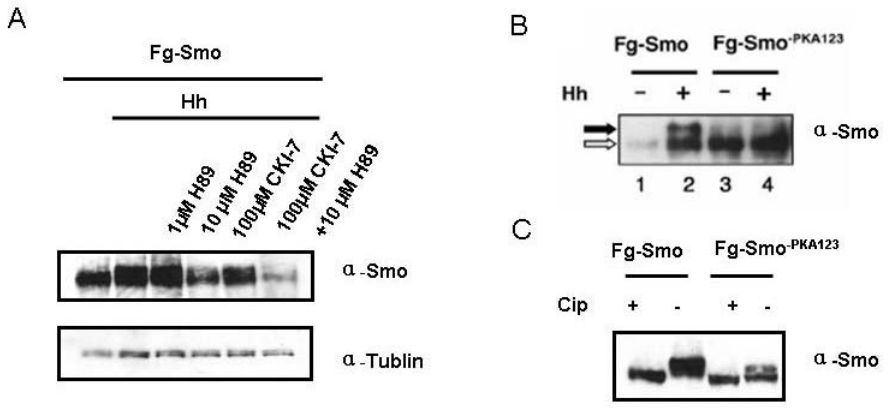



Fig 3.2- Hh induced Smo phosphorylation is PKA and CKI activity dependent. (A) S2 cells transfected with Fg-Smo were treated with or without Hh in presence or absence of PKA and/ or CKI inhibitor as indicated. Cell extracts were immunoprecipitated by anti-Fg antibody, followed by immunobloting with anti-SmoC antibody. Hh induced Smo mobility shift and Smo accumulation were greatly reduced with the addition of drugs. 10% total cell extracts were also analyzed by immunoblotting with anti-Tubulin antibody to indicate the equal extract inputs for the immunoprecipitation. (B) S2 cells transfected with Fg-Smo or Fg-Smo$^{-PKA123}$ were treated with or without Hh conditional medium. Cell extracts were immunoprecipited with anti-Fg antibody and analyzed by western bolt with anti-SmoC antibody. Fg-Smo phosphorylation was indicated by the slow mobility band labeled by the black arrow. (C) Wing discs expressing UAS-Fg-Smo or UAS-Fg-Smo$^{-PKA123}$ with *MS1096* were lysised and then treated with or without CIP as indicated. Cell extracts were immunoprecipitated with anti-Fg antibody, followed by immunoblotting with anti-Smo C antibody. The collapse of the slow mobility bands with CIP treatment indicated the slow migrating forms of Smo are hyperphosphorylated.

To determine whether Hh induced Smo phosphorylation at PKA sites, Fg-Smo and Fg-Smo$^{-PKA123}$ were transfected to S2 cells, followed by treating with Hh conditional medium for 24 hours. Then the cell extracts were immunoprecipited with mouse anti-Fg antibody, followed by western blot analysis with mouse anti-Fg antibody. As shown in Fig. 3.2B, Hh induced the appearance of slow migrating, hyperphosphorylated forms of Fg-Smo. In contrast, Fg-Smo$^{-PKA123}$ did not exhibit significant mobility shift in response to Hh. When overexpressed in wing imaginal discs by using *MS1096* Gal4 driver, Fg-Smo, which is mainly derived from P-compartment cells, existed in slow migrating hyperphosphorylated forms, indicated by the collapse of the shift bands after the treatment of calf intestine phosphatase (CIP). In contrast, the majority of Fg-Smo$^{-PKA123}$ existed in fast migrating, nonphosphorylated forms. However, a small amount of Fg-Smo$^{-PKA123}$ could undergo low levels of phosphorylation, indicating that there are other phosphorylation sites in Smo C-tail in addition to those PKA and CKI sites we identified. Taken together, these data suggest that Hh induces phosphorylation of Smo by PKA and CKI at multiple sites in Smo C-tail.

Smo phosphorylation is essential and sufficient for its cell surface expression and activity.

To determine if Smo phosphorylation is essential for Hh pathway activation, Jianhang *et al.* assessed the activity of Flag-tagged wild type or Smo variants with one, two, or three PKA sites mutated to Ala (Jia et al., 2004). Overexpression Fg-Smo induced ectopic albeit weak Hh pathway activation, as evidenced by low levels of ectopic *dpp* expression in the anterior cells far away from A/P boundary (Fig. 3.3a). Fg-Smo$^{-PKA3}$ induced ectopic *dpp* expression at the level similar to wild-type Smo (Fig. 3.3b) and

restored Hh signaling activity in *smo* mutant cells at the A/P boundary (data not shown).

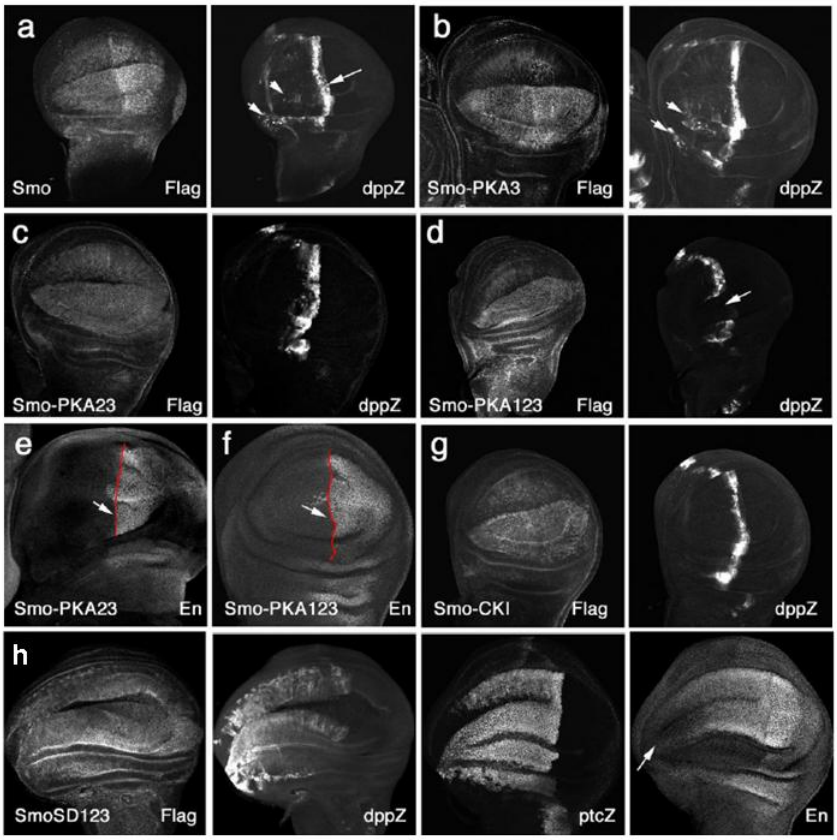



Fig. 3.3- Hh signaling activity of Smo variants (This figure is contributed by Jianhang Jia). a-d, g, Wing discs expressing Fg-Smo(a), Fg-Smo$^{-PKA3}$ (b), Fg-Smo$^{-PKA23}$ (c), Fg-Smo$^{-PKA123}$ (d), and Fg-Smo$^{-CKI}$ (g) with *MS1096* were stained with anti-Fg and anti-lac*Z* antibody to show Flag and *dpp-lacZ* expression. Arrow head in (a) and (b) indicates the ectopic *dpp* expression. Arrow in (a) shows the normal expression pattern of *dpp* which is block by Fg-Smo$^{-PKA123}$ expression, indicated by arrow in (d). e, f, Wing discs expressing Fg-Smo$^{-PKA23}$ and Fg-Smo$^{-PKA123}$ were immunostained to show Flag and *en* expression. En expression were blocked by Fg-Smo$^{-PKA23}$ and Fg-Smo$^{-PKA123}$ as indicated by the arrow in e and f. h, Wing discs expressing Fg-Smo$^{SD123}$ with *MS1096* were stained to show Flag, *dpp-lacZ*, *ptc-LacZ* and En expression. The arrow showed that En is not ectopicly expressed in the anterior cells away from A/P boundary.

Although Fg-Smo$^{-PKA23}$ still could restore dpp expression and partially restore *ptc* expression near the A/P boundary in *smo* mutant cells, it cannot ectopic turn on dpp expression (Fig. 3.3c) and it blocked *en* expression at the A/P border (Fig. 3.3e). These

data suggested that Fg-Smo$^{-PKA23}$ could transduce low but not high levels of pathway activity. In contrast, Fg-Smo$^{-PKA123}$, failed to rescue any Hh target gene expression in *smo* mutant cells (Fig. 3.3f). In addition, Fg-Smo$^{-PKA123}$ blocked the expression of Hh target gene including *dpp-LacZ* expression at the A/P boundary (Fig. 3.3d), suggesting that it not only failed to transduce Hh signal but also interfered with endogenous Smo. Hence, a single PKA site seems to be necessary for low levels of pathway activity whereas two or more PKA sites appear to be essential for high levels of pathway activity.

Fg-Smo$^{-CKI}$ also failed to induce ectopic *dpp* expression (Fig. 3.3g), and did not rescue Hh target gene expression in *smo* mutant cells, suggesting that CKI sites phosphorylation is also essential for Smo activity.

To determine whether phosphorylation of Smo on PKA and CKI sites is sufficient to activate Hh signaling pathway, we substituted the PKA and CKI sites to acidic residue Asp to mimic phosphorylation and constructed Flag tagged Smo variant Fg-Smo$^{SD123}$ (Fig. 3.1A). Strikingly, Fg-Smo $^{SD123}$ induced ectopic expression of high levels of *ptc* and *en* (Fig. 3.3h). The constitutive activity of Fg-Smo $^{SD123}$ is independent of endogenous of Smo (Jia et al., 2004). These observations suggested that phosphorylation on PKA and CKI sites is sufficient to activate Hh responses. However, Fg-Smo $^{SD123}$ did not induce ectopic *en* expression in anterior-most region of wing discs, implying that its activity could still be modulated by Hh.

Previous studies suggested that the interaction between Smo and downstream Cos2-Fu complex is essential for Hh signal transduction. To test if the phosphorylation on Smo affects the physical interactions between Smo and Cos2-Fu complexes, we transfected S2 cells with Fg-Smo or Fg-Smo$^{-PKA123}$ together with HA-Cos2 and Myc-Fu with or without

Hh stimulation. The cell extracts were immunoprecipited with mouse anti-Flag antibody, followed by western blot analysis. As shown in Fig 3.4A, Fg-Smo and Fg-Smo$^{-PKA123}$ pulled down comparable amount of Cos2 and Fu, indicating the phosphorylation on Smo does not dramatically changing the binding affinity between Smo and Cos2-Fu complex. These data may provide an answer to why Fg-Smo$^{-PKA123}$ has dominant negative effects on Hh downstream gene expression at the A/P border. Since Fg-Smo$^{-PKA123}$ interacts with Cos2-Fu complex with the similar affinity as the wild-type Smo, Fg-Smo$^{-PKA123}$ overexpressing should titrate out the available Cos2 and Fu and therefore interferes with Hh responses mediated by the endogenous Smo. Indeed, when we transfected cells with the same amount of Fg-Smo expressing construct and increase amount of GFPSmo$^{-PKA123}$ expressing construct together with Cos2 and Fu, the amount of Cos2 and Fu pulled down by Fg-Smo is decreased (Fig. 3.4B).

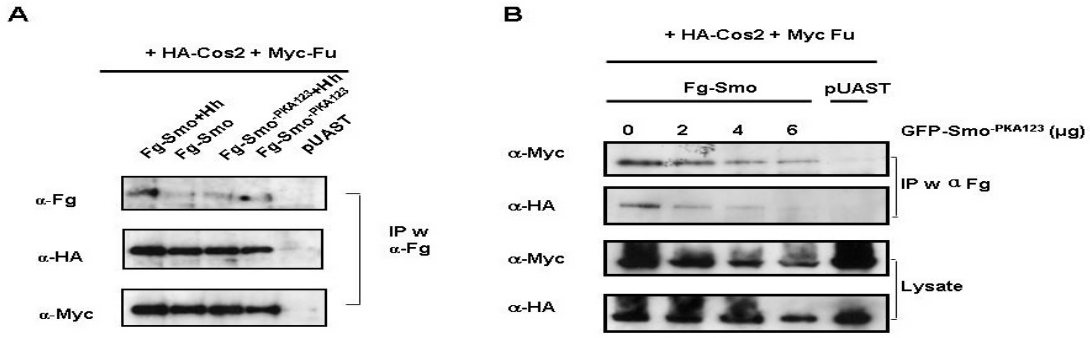



Fig. 3.4- Phosphorylation does not affect the interaction affinity between Smo and Cos2-Fu complex. (A) S2 cells transfected with Fg-Smo (lane 1, 2) or Fg-Smo$^{-PKA123}$ (lane 3, 4) in conjugation with HA-Cos2 and Myc-Fu were treated with or without Hh as indicated. Cell extracts were immunoprecipitated (IP) with anti-Fg antibody, followed by westernblotting with antibodies as indicated. Fg-Smo and Fg-Smo$^{-PKA123}$ pulled down similar amount of Cos2 and Fu. (B) GFP-Smo$^{-PKA123}$ competed with Fg-Smo for interacting with Cos2 and Fu. S2 cells were transfected with 2μg Fg-Smo expressing constructs and increase amount of GFP-Smo$^{-PKA123}$ expressing construct as indicated together with HA-Cos2 and Myc-Fu. Cell extracts were immunoprecipated with anti-Fg antibody, followed by immunoblotting with antibodies as indicated. 5% total cell extracts were also analyzed by western blotting with indicated antibodies.

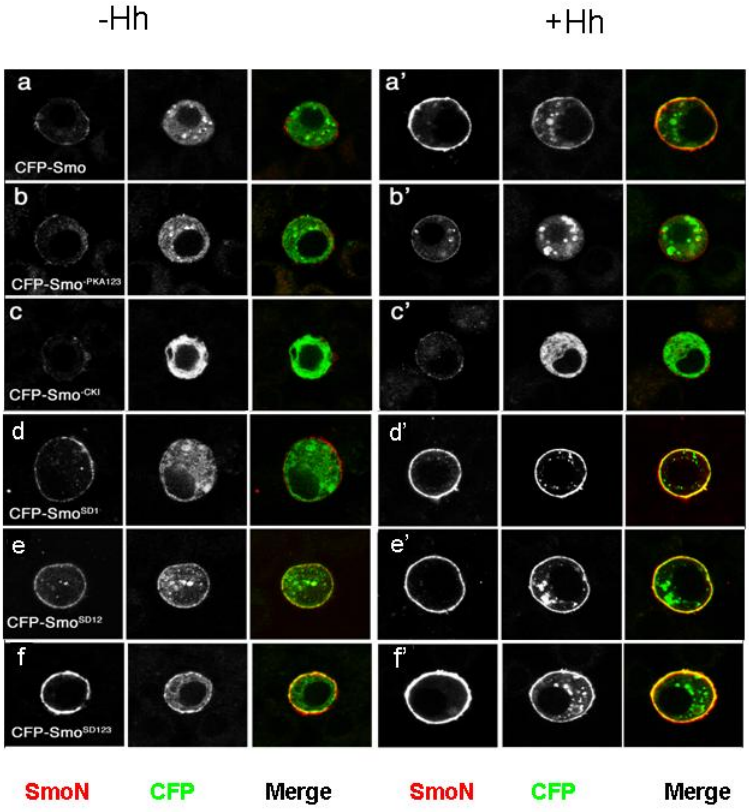



Fig. 3.5- Regulation of Smo cell surface accumulation by phosphorylation. S2 cells transfected with CFP-Smo (a, a'), CFP-Smo$^{-PKA123}$ (b, b'), CFP-Smo$^{-CKI}$ (c, c'), CFP-Smo$^{SD1}$ (d, d'), CFP-Smo$^{SD12}$ (e, e') and CFP-Smo$^{SD123}$ (f, f'), treated with (a'-f') or without (a-f) Hh, were stained with anti-Smo-N antibody under non-permibilized condition. The levels of cell surface Smo were indicated by the red signal and the total levels of Smo were indicated by the green signal. PKA and CKI sites mutation abolished Hh induced Smo cell surface accumulation. Mimic phosphorylation promotes Smo cell surface expression. The basal levels of cell-surface CFP-Smo$^{SD1}$ and CFP-Smo$^{SD12}$ were higher than that of CFP-Smo but lower than that of CFP-Smo$^{SD123}$.

The level of Fg-Smo is higher in posterior cells, however, the levels of Fg-Smo$^{-PKA123}$ and Smo$^{-CKI}$ fail to elevated in posterior compartment (Jia et al., 2004), implying that phosphorylation may be required for Hh induced Smo cell surface accumulation. To test this hypothesis, we transfected S2 cell with CFP tagged wild type or various mutant forms of Smo and stained cells with anti-Smo N terminus antibody without cell

permibilization to visualize the cell surface Smo levels. The total Smo expression level was observed by the CFP auto-fluorescence. As showed in Fig. 3.5, CFP-Smo exhibited low levels of cell surface expression in the absence of Hh, but accumulated on cell surface in response to Hh. Neither CFP-Smo$^{-PKA123}$ nor CFP-Smo$^{-CKI}$ accumulated on cell surface regardless of Hh treatment. Strikingly, CFP-Smo$^{SD123}$ exhibited high levels of cell surface expression in quiescent cells without Hh treatment, which may account for its constitutive activity *in vivo*. These data suggest that phosphorylation of Smo is essential and sufficient for Smo accumulated on cell surface.

The level of Smo phosphorylation correlates the level of Smo cell surface expression and activity.

Since there are multiple phosphorylation sites in Smo C-tail, it is interesting to know if differential phosphorylation on Smo results in graded Smo activity. To test this, Jianhang *et al.* used *MS1096* Gal4 driving either Fg-Smo$^{SD1}$ or Fg-Smo$^{SD12}$ overexpressing in wing imaginal discs and assayed the ectopic downstream gene expression in the anterior cells far away from A/P boundary. Interestingly, Fg-Smo$^{SD1}$ induced higher level of *dpp* expression than Fg-Smo, but failed to induce *ptc* and *en* expression. Fg-Smo$^{SD12}$ induced ectopic albeit low levels of *ptc* expression, but failed to ectopicly turn on *en* expression (Jia et al., 2004). These data indicate that the levels of Smo activity appear to correlate with its levels of phosphorylation.

To test if the phosphorylation levels of Smo also affect Smo cell surface accumulation, we transfect S2 cell with CFP-Smo$^{SD1}$ and CFP-Smo$^{SD12}$ and observed their cell surface level. As showed in Fig 3.5, the basal cell surface levels of Smo$^{SD1}$ and Smo$^{SD12}$ are higher than wild-type Smo but still lower than Smo$^{SD123}$. These data

suggested that the levels of Smo cell surface expression also correlates with its levels of phosphorylation.

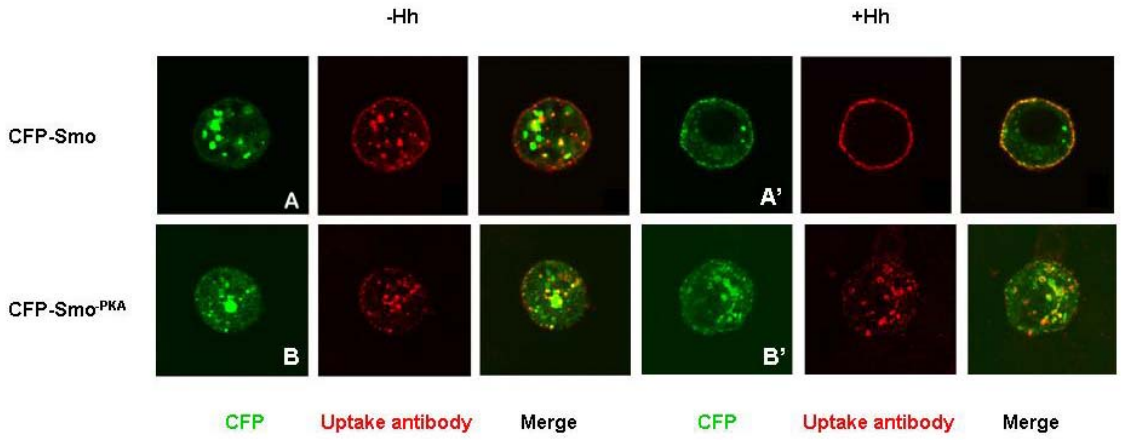



Fig. 3.6- Phosphorylation regulates Smo cell surface expression by regulating Smo trafficking. S2 cells transfected with CFP-Smo (A, A') or CFP-Smo$^{-PKA123}$ (B, B'), treated with (A'-B') or without (A-B) Hh, were incubated with anti-SmoN antibody in S2 medium to allow internalization of cell surface bound antibodies. Internalization of CFP-Smo but not CFP-Smo$^{-PKA123}$ was largely inhibited by Hh stimulation.

Phosphorylation of Smo could regulate its intracellular trafficking and/or stability on the cell surface. To address this, we carried out an antibody uptake experiment. S2 cells were incubated in the medium containing anti Smo-N antibody for 30 minuets and then washed with PBS twice, followed by fixing, permibilization and incutating with secondary antibody. Internalized CFP-Smo was readily detected in quiescent cells (Fig. 3.6A), indicating that CFP-Smo could reach the cell surface but was removed by endocytosis. Upon Hh stimulation, internalized CFP-Smo was greatly reduced and CFP-Smo accumulated on the cell surface (Fig. 3.6A'). In contrast, internalized CFP-Smo$^{-PKA123}$ was detected regardless of Hh stimulation (Fig. 3.6B, B'), indicating that

phosphorylation may regulate Smo cell-surface expression, at least in part, by inhibiting endocytosis and/or promoting recycling.

## Discussion

In summary, our data suggest that PKA and CKI phosphorylate Smo at multiple sites to promote its cell surface accumulation and signaling activity in response to Hh. The good correlation between cell surface localization and signalling activity suggests that phosphorylation induced cell surface accumulation of Smo is likely to contribute to Hh pathway activation.

One kinase, two tales

PKA is one of the best characterized protein kinase family member (Kim et al., 2006). The inactive PKA holoenzyme complex includes two fully phosphorylated catalytic subunits (C) and two regulatory subunits (R). The activation of PKA is achieved by interaction of cAMP with the regulatory subunit and releasing the active catalytic subunits. The substrate specificity is often achieved by the targeting of the holoenzyme to specific sites via A-kinase-anchoring proteins (AKAPs)(Kim et al., 2006). The CKI family of serine/threonine kinase is involved in many diverse and important cellular functions (Gross and Anderson, 1998; Knippschild et al., 2005; Price, 2006). CKI has been identified in the entire eukaryotic organism from yeast to human. In mammals, there are seven members in CKI family: α, β, γ1, γ2, γ3, δ, and ε (Hanks and Hunter, 1995). In *Drosophila* there are eight CKI family members (Morrison et al., 2000). PKA and CKI are involved in both inhibition and activation of Hh pathway (Jia et al., 2002; Price and Kalderon, 2002). The positive roles and the negative roles of PKA and CKI appear to be switched by Hh stimulation. In the absence of Hh, PKA phosphorylates Ci155 at three defined sites and primes Ci for further phosphorylation by CKI and GSK3, which is essential for Ci processing (Jia et al., 2002; Price and Kalderon, 2002). Here, we found

that PKA phosphorylats Smo and prims it further phosphorylated by CKI in three phosphorylation clusters in response to Hh and the phosphorylation is critical for Smo activity.

It is still not clear how PKA and CKI switch targets in response to Hh. Our group showed that PKA and CKI associate with Cos2 and promote Ci155 phosphorylation in the absence of Hh (Zhang et al., 2005). In response to Hh, these kinases dissociate from Cos2 and Ci, leading to diminished Ci phosphorylation. It is likely that PKA and CKI interact with Smo afterwards and phosphorylate Smo. Consistent with this prediction, we found that Smo and PKA are in the same complexes (Fig. 3.7). However, we could consistently detect PKA by immnoprecipitating Smo, regardless of Hh stimulation. In the absence of Hh, the interaction between PKA and Smo could be mediated by Cos2. With Hh stimulation, PKA may dissociate from Cos2 and directly interact with Smo. Yeast two hybrid and FRET assay may help to test these possibilities.

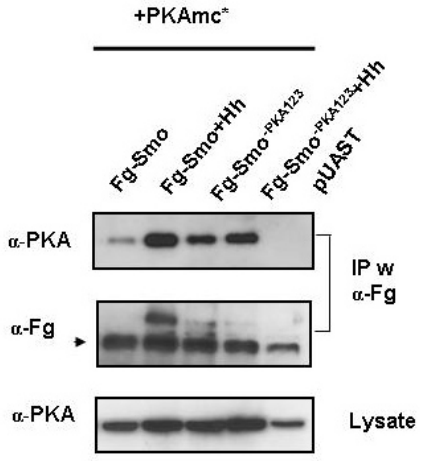



Fig. 3.7- Smo interacts with PKA catalytic subunit. S2 cells transfected with Fg-Smo or Fg-Smo$^{-PKA123}$ in conjugation with PKAmc* were treated with or without Hh. Cell extracts were immunoprecipated by anti-Fg antibody, followed by immunoblotting with antibodies as indicated. The arrow indicates a non-specific band. 5% total cell extracts were also analyzed by Westernblotting with anti-PKA antibody. Both Fg-Smo and Fg-Smo$^{-PKA123}$ interact with PKA catalytic domain. The increase of Fg-Smo and PKA interaction in presence of Hh reflects the increase of Fg-Smo in response to Hh

Using the same set of kinases to regulate a signaling pathway both positively and negatively seems to be odd. However, this kind of strategy is not unique to Hh pathway. It has been shown recently that multiple CKI family members are involved both

positively and negatively in regulating Wnt signaling pathway (Price, 2006). Several components such as Dvl, LEF/TCF and the degradation complex in Wnt pathway has been indicated as the potential targets of CKI family kinases (Price, 2006). The phosphorylation of these components by CKI results in inhibiting GSK3 activity and stabilizing β-Catenin (Yost et al., 1998) (Lee et al., 1999; Li et al., 1999). Recently, it has been shown that CKIγ, a membrane tethered form CKI, is involved in phosphorylation of Wnt coreceptor LRP6 (Arrow)(Davidson et al., 2005; Zeng et al., 2005). Upon phosphorylation, LRP6 recruits the scaffold protein Axin and blocks the degradation of β-Catenin and therefore activates Wnt pathway. Addition to these positive roles, CKI family member also has negative roles in Wnt pathway. CKIα phosphorylates β-Catenin which promotes β-Catenin phosphorylation by GSK3 and leads to β-Catenin degradation (Liu et al., 2002; Yanagawa et al., 2002). In addition to β-Catenin, APC is another substrate of CKI (Gao et al., 2002; Ha et al., 2004; Kishida et al., 2001; Xing et al., 2004). The phosphorylation of APC by CKIδ and CKIε also leads to the degradation of β-Catenin and inhibit Wnt pathway.

Interestingly, different CKI family members respond to Wnt signaling differently. Wnt activates CKIε by dephosphorylating CKIε (Swiatek et al., 2004). However, in the case of LRP6 phosphorylation in responce to Wnt, it is likely that the exposure of LRP6 phosphorylation region but not the CKIγ activity is regulated by Wnt (Davidson et al., 2005; Zeng et al., 2005). Whether Wnt signaling inhibits CKIα activity is still unclear. The differential regulation of different CKI family members may provide us some hints about how Smo and Ci phosphorylation are regulated by Hh. Both CKIε (double time) and CKIα appear to be involved in Ci and Smo phosphorylation (Jia et al., 2005). It will

be interesting to know if Hh signaling affects the activity of these kinases. In the case of Ci phosphorylation, it is likely that the subcellular localization of PKA and CKI rather than the activity of these kinases is regulated by Hh (Zhang et al., 2005).

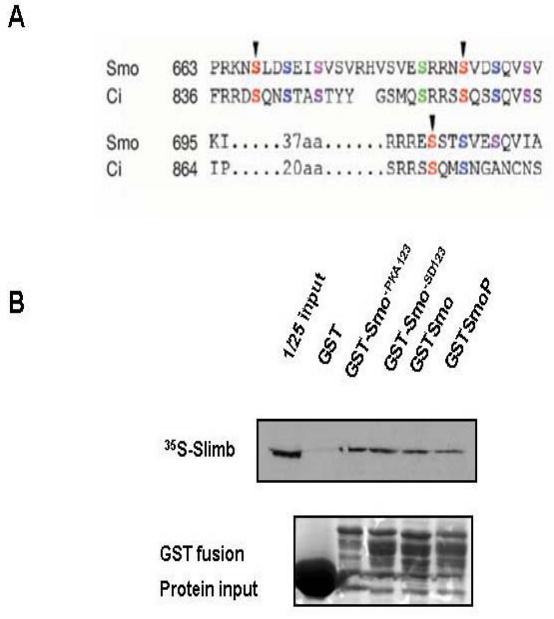



Fig. 3.8 - The comparison of the phosphorylation clusters between Smo and Ci. (A) (Adopted from Apionishev *et al*. 2005). The aligment of the phosphorylation cluster in Smo and Ci. Red, blue (purple), and Green Ser indicate PKA, CKI and GSK sites, respectively. (B) Slimb interacts with Smo weakly regardless of Smo is phosphorylated or not. [$^{35}$-S] labeled *in vitro* translated Slimb was incubated with GST fusion Smo variants as indicated. The interaction was detected by SDS-PAGE followed by autoradiography (upper panel). The GST protein inputs were shown by SDS-PAGE followed by Commassie Blue staining. GSTSmo phosphorylated by PKA and CKI *in vitro* was indicated as GSTSmoP.

There is a predicted GSK3 phosphorylation site in Smo C-tail. However, the S to A mutation in that site does not change Smo activity dramatically, indicating GSK3 probably was not involved in Smo activity regulation (Apionishev et al., 2005). Interestingly, the phosphorylation clusters in Ci155 and Smo are quite similar to each other (Fig. 3.8 A), indicating the similar regulation strategy may be applied by both proteins. The phosphorylation clusters in Ci155 recruit Slimb upon PKA, CKI and GSK3 phosphorylation (Jia et al., 2005). However, Slimb appears to weakly interact with Smo regardless Smo is phosphorylated or not (Fig. 3.8 B). Furthermore, phosphorylation on Ci promotes Ci processing, while, phosphorylation on Smo appears to stabilize Smo.

These data suggest that Ci and Smo may be regulated differently by phosphorylation, although they have similar phosphorylation clusters.

How is Smo phosphorylation regulated?

In this study, we found Smo is phosphorylated by PKA and CKI at three phosphorylation clusters in response to Hh. Then the question becomes how Smo phosphorylation is regulated.

One possible model is that the activation of kinases by Hh leads to Smo phosphorylation. The canonical PKA activation model is that cAMP binds to the regulatory subunits of PKA and releases the catalytic subunits from the regulatory subunits, and thereby activates PKA. However, the manipulation of cAMP concentration does not seem to affect Hh signaling activity (Li et al., 1995). In addition, we found simply overexpression of mC* cannot *de novo* activate Hh pathway and Hh further boosts the activation of Smo by mC*. These data suggests that PKA activation is not sufficient to activate Hh pathway. However, one cannot exclude the possibility that Hh regulates PKA activity through other means and PKA activation may contribute to Hh induced Smo phosphorylation. But, until now the evidence of PKA activation in response to Hh is still lacking.

The other possible model is Hh stimulation may change Smo conformation and expose the phosphorylation sites to PKA and CKI. In the absence of Hh, Smo is inhibited by Ptc by an unknown mechanism (Ingham and McMahon, 2001). Currently, the most favorite model is the "pump model" (Taipale et al., 2002). Ptc consistently pumps out Smo agonist and Hh inhibits this process. The accumulation of against inside cells leads to activate Smo. It is possible that the interaction between Smo agonist and Smo changes

Smo C-tail conformation and exposes the phosphorylation clusters and therefore promotes Smo activation. However, there is no such small molecule Smo agonist has been identified in the physiological condition. In addition, Smo phosphorylation happens so fast, it is hard to analyze the conformation change prior to Smo phosphorylation in response to Hh if there is any. Ideally, there are some mutated forms of Smo which still keep the ability to undergo conformational change in response to Hh but lack the phosphorylation sites. Then we could observe the response of Smo to Hh stimulation prior to its phosphorylation.

The third possible model for Smo phosphorylation is that Smo is consistently phosphorylated but the phosphorylation was reversed by phosphatase activity. In response to Hh, the activity of the phosphatase is inhibited, which leads to Smo phosphorylation and activation. However, there is no direct evidence suggests that phosphatases are involved in Smo phosphorylation regulation. In a large scale RNAi screen carried in *Drosophila* cl8 cells, some phosphatases were identified as positive regulators in Hh pathway (Nybakken et al., 2005). It is possible due to their effects on Ci phosphorylation. The studies carried on so far cannot exclude the possibilities that phosphatases participate in Smo phosphorylation regulation.

In addition to all those models discussed above, it is possible that the availability of the kinases to Smo may also regulate Smo phosphorylation. In the absence of Hh, PKA and CKI interact with Cos2 (Zhang et al., 2005), which may prevent those kinases from getting access to Smo. Although Smo and a small amount of Cos2 are constitutively interact with each other regardless of Hh stimulation (see chapter two), the Cos2-Kinases complex and Smo-Cos2 complex may not be the same complex. Even they are in the

same complex, the manner of Smo-Cos2 interaction may mask the phosphorylation sites to prevent Smo from being phosphorylated by those kinases. In response to Hh, Cos2 may change conformation to expose the phosphorylation sites.

Phosphorylation regulates Smo cell surface accumulation.

In this study, we found that phosphorylation of Smo in three defined clusters stabilizes Smo and promotes its cell surface accumulation by reducing Smo endocytosis or encouraging its recycling. However, it is still not clear how phosphorylation stabilizes Smo and how phosphorylation regulates Smo trafficking.

It has been shown that Smo mainly localized in lysosomes in the absence of Hh (Nakano et al., 2004). With Hh stimulation, the majority of Smo is accumulated on the cell surface and a small part of Smo is localized in the early and late endosomes (Nakano et al., 2004). In *hrs* (a gene encodes a sorting protein that associates with endosome and binds to ubiquitin) mutant clones, Smo accumulates in compartments highly enriched in ubiquitinated proteins (Jekely and Rorth, 2003), implying ubiquitination may be involved in Smo trafficking regulation. It is possible that Smo recruit some E3 ligase which promotes Smo ubiquitination and drives Smo degradating in lysosomes. In response to Hh, Smo is phosphorylated, which may dissociate Smo from the E3 and promote Smo recycling. However, there is no direct evidence Smo is ubiquitinated.

A distinct feature of Smo phosphorylation is that the phosphorylation sites form clusters which create several acidic stenches in Smo C-tail. It has been reported that some acidic amino acid clusters in the G protein-activated inwardly rectifying potassium channels (Kir3, GIRK) are important to maintain high levels of cell surface expression of those channels (Ma et al., 2002a). Deletion or mutation of those sequences resulted in

greatly reduction of the cell surface levels of the channels, with the corresponding increase of the channels in late endosomes and lysosomes (Ma et al., 2002a). It has been indicated that the acidic clusters probably promotes channels cell surface expression by promoting the recycling from early endosome or by retrieval of channels from endosome to tran-Golgi network (TGN), where the channels could then be target to cell surface. A protein named PACS-1 has been shown to recognize the acidic sequences and retrieval a variety of proteins to TGN (Crump et al., 2001; Wan et al., 1998). It is possible this protein may interact with phosphorylated Smo and promotes its cell surface accumulation. I had some preliminary data showing that KrT95D, the ortholog of PACS-1 in *Drosophila*, interacted with Smo weakly regardless Smo is phosphorylated or not, making it unlikely that PACS-1 is involved in regulating Smo trafficking. However, it is still possible that other sorting protein may recognize the phosphorylation clusters in Smo C-tail and promote Smo cell surface accumulation.

Phosphorylation regulates Smo activity

In this study, we found that Smo is phosphorylated at multiple phosphorylation clusters in response to Hh, which is necessary and sufficient to activate Smo. However, it is still not clear how phosphorylation regulates Smo activity.

Although cell surface accumulation may contribute to overall Smo activity, phosphorylation may activate Smo through other means. One possiblility is that phosphorylation may change Smo C-tail conformation and therefore change the interaction mode between Smo and Cos2-Fu complex. Smo may interact with Cos2-Fu complex in a manner that may promote phosphorylation of Cos2 and Fu and therefore counteract the negative effects of Cos2 and Sufu on Ci. By using antibody epitope

exposure assay, Ingham's group suggested that Smo C-tail undergoes conformational change in response to Hh (Ingham et al., 2000). It is possible that phosphorylation on Smo C-tail mediates this conformational change. FRET analysis may reveal some new aspects of Smo-Cos2 interaction. In addition, it has been implicated that dimmerization of Smo is likely to activate Smo; therefore, it will be interesting to test whether Smo phosphorylation activates Smo by promoting its dimmerization.

In this study, our data showed that the levels of Smo activity are correlated with the levels of Smo phosphorylation, indicating that differential phosphorylation of Smo may gradually increase Smo activates. As a morphogen, different Hh concentrations regulate different cellular responses. It is interesting to know whether different levels of Hh result in differential phosphorylation of Smo CT. We hypotheses that low levels of Hh induce low levels of Smo phosphorylation, leading to low threshold target gene expression. In response to high levels of Hh stimulation, the levels of Smo phosphorylation increase, which induces high threshold target gene expression. Raising site specific phosphorylation antibodies may help answer this question.

## Materials and Methods

Plasmid construction and transgenes

*smo3* and *DC0E95* are null alleles. *UAS-mC**, *UAS-R**, *MS1096*, *ap-Gal4, dpp-lacZ and ptc-lacZ* have been described. Smo variants with substitutions at PKA and CKI sites were generated by site-directed mutagenesis. A Flag tag or CFP was fused at the C-terminus of each Smo variant, followed by subcloning into pUAST. Five or more independent transformant lines were examined for each Smo variant. To construct *UAS-CKIα RNAi*, a genomic DNA fragment with coding sequence for CKIα aa153-337 was cloned into pUAST with the corresponding cDNA fragment inserted in a reverse orientation. To construct pGEXSmo656-755, pGEXSmo656-755$^{-PKA123}$, and pGEXSmo656-755$^{-CKI}$, *smo* cDNA fragments encoding Smo aa656-755 and corresponding variants with PKA and CKI sites mutated were amplified by PCR and inserted between NotI and EcoRI sites on the pGEX4T-2 vector.

Clones of mutant cells were generated by *FLP/FRT*-mediated mitotic recombination as described. Genotypes for generating clones are as follows. *DC0* clones: *y hsp-flp; DC0E95 stc FRT40/ hsp-Myc-GFP FRT40. smo-* clones expressing Smo variants: *y hsp-flp/MS1096; smo3 FRT 39E/hsp-CD2, y+ FRT39E; UAS-Fg-Smo-PKA(CKI)/dpp-lacZ or ptc-lacZ.*

Cell culture and transfection and drug treatment

Cell culture and transfection were performed as described in chapter two. PKA inhibitor H89 (Calbiochem) and CKI inhibitor CKI-7 (Upstate) were dissolved in DMSO at the concentration 10mM and 100mM, respectively. Transfected cells were incubated

with H89 or/and CKI-7 for 4 hours before harvest. The final concentration of H89 is 1μM or 10μM. The final concentration of CKI-7 is 100μM.

Immunoprecipitation, Western blot analysis, and *in vitro* kinase assay

Immunoprecipitation and western blot analysis were performed as described in chapter two. GST fusion proteins were made with standard protocols. Briefly, pGEX expression constructs were transformed into *E.coli* BL21 strain and protein expression was induced by 80uM IPTG for 6 hours at room temperature. The bacteria were harvested and sonicated in PBS buffer with 10% Glycerol. The bacteria lysate was incubated with Glutathione Sepharose beads (Amersham) for 1 hour and followed by washing with PBS for three times. The amounts of different proteins were normalized by adjusting the volume of the lysates according to the protein quantity indicated by protein purified from 100ul of lysates. To perform the *in vitro* kinase assay, beads bound GST fusion proteins were washed by 1× kinase buffer and incubated with the recombinant kinase PKAc or CKIα (NEB) and [$\gamma$-$^{32}$P]ATP for 2 hours, followed by running SDS-PAGE. Gels were dried and the extent of phosphorylation was assayed by autoradiography.

Immunostaining, cell surface accumulation assay, and antibody uptake assay

Immunostaining of imaginal discs was done with standard protocols. Briefly, wing imaginal discs of third in star larvae were dissected and fixed by 4% formaldehyde in PBS for 20 minuets, followed by washing with PBT (PBS with 0.1% Triton X-100) for three times. Then the discs were incubated with primary antibody overnight at 4°C, followed by washing with PBT for three times and incubating with secondary antibody for 2 hours at room temperature. Then the discs were washed three times with PBT and

mounted on the slides in 80% Glycerol. For cell surface staining, transfected cells were fixed with 4% paraformaldehyde for 15 minuets and incubated with primary antibody in PBS for 30 min at room temperature, followed by washing with PBS for three times and incubating with secondary antibody for 1 hour at room temperature. For antibody uptake, transfected cells were incubated with primary antibody in S2 cell medium for 30 minutes at room temperature, followed by fixation, permeabilization, and secondary antibody staining. Antibodies used in this study: rat anti-Ci (2A)(Motzny and Holmgren, 1995), mouse anti-En (DSHB), rabbit anti-Col (Vervoort et al., 1999), rabbit anti-β Gal (Cappel), mouse anti-CD2 (Serotec), mouse anti-Flag (Sigma), rat anti-SmoC (Denef et al., 2000), and mouse anti-SmoN (DSHB).

# CHAPTER FOUR

## Multiple Arginine clusters in Smo C tail negatively regulate Smo activity

### Introduction

The Hedgehog morphogen controls distinct cell fates through graded signaling activity (Alexandre et al., 1996; Strigini and Cohen, 1997). In chapter three, we identified several phosphorylation clusters in Smo C-tail and demonstrated that phosphorylation in these clusters is critical for Smo cell surface accumulation and activity in response to Hh. Our data also implied that Smo may relay different levels of Hh activity through differential phosphorylation of its C-tail, however, how phosphorylation regulates Smo cell surface expression level and activates Smo is largely unknown.

Previous studies by our lab have shown that MyrSmoCT but not the soluble form SmoCT contains constitutive low level of Smo activity (Jia et al., 2003), indicating that the membrane tethering of Smo is essential for its activity. Our recent study also showed that the cell surface levels of Smo correlated with its activity, suggesting that cell surface accumulation of Smo may be important for Smo activity. To understand how phosphorylation regulates Smo cell surface expression, I have performed cell surface expressing assay for a series C-terminal truncation forms of Smo and in an attempt to identify domians in Smo that regulate Smo cell surface accumulation and Smo activity.

It has been shown that Smo C-tail may undergo conformational change in response to Hh (Ingham et al., 2000). It is possible that phosphorylation of Smo C-tail

may mediate such conformational change to activate Smo. It is interesting to know if phosphorylation on Smo C-tail promotes Smo undergoing conformational change and activate Smo.

In this study, we identify multiple arginine (Arg) clusters in SmoC-tail that negatively regulates Smo activity and demonstrate that Smo activity is inversely correlated with the number of Arg clusters. We show that Arg clusters inhibit Smo by blocking its cell surface accumulation and keeping SmoC-tail in a "closed", inactive conformation maintained by intracellular electrostatic interactions. We provide evidence that phosphorylation antagonizes the negative regulation of Arg clusters in part by neutralizing their positive charges, which disrupts intracellular electrostatic interactions and allows SmoC-tail to adopt an "open", active conformation. We propose that multiple Arg clusters endow Smo the ability to respond to different levels of Hh through differential phosphorylation.

## Results

Multiple Arginine clusters in Smo C-tail negatively regulate Smo cell surface expression and activity.

To understand how Smo cell surface expression is regulated, we developed a cell base assay to dissect whether there is any cis-element on Smo C-tail which regulates Smo cell surface accumulation. We transfected S2 cell with CFP tagged Smo variants and stained the transfected cells with anti-SmoN antibody before membrane permeabilization to visualize cell surface localized Smo. SmoΔC570 (Fig. 4.1a, c), a truncated form of Smo with most of its C-tail deleted, showed dramatic cell surface accumulation, suggesting that negative element(s) may exist in the Smo C tail to prevent Smo cell surface accumulation.

To identify the negative element(s), a series of CFP tagged Smo deletion mutants were generated (Fig. 4.1a), and the cell surface expression of these mutants was detected. As shown in Fig. 4.1, the short deletions up to aa818 (SmoΔC860, SmoΔC818) of Smo C-tail did not significantly change cell surface expression (Fig. 4.1 l, m). However, further deletions resulted in progressive increase in cell surface levels of corresponding Smo variants (SmoΔC750, SmoΔC730, SmoΔC661, SmoΔC570) (Fig. 4.1 k, j, e, c). There was a dramatic difference in cell surface level between SmoΔC661 and SmoΔC818 (comparing Fig. 4.1 e with l), indicating that the region between aa661 to aa818 includes a negative element(s) preventing Smo cell surface accumulation. Consistently, deletion of this region (SmoΔ661-818) resulted in high levels of cell surface expression of corresponding Smo variant (Fig. 4.1o).

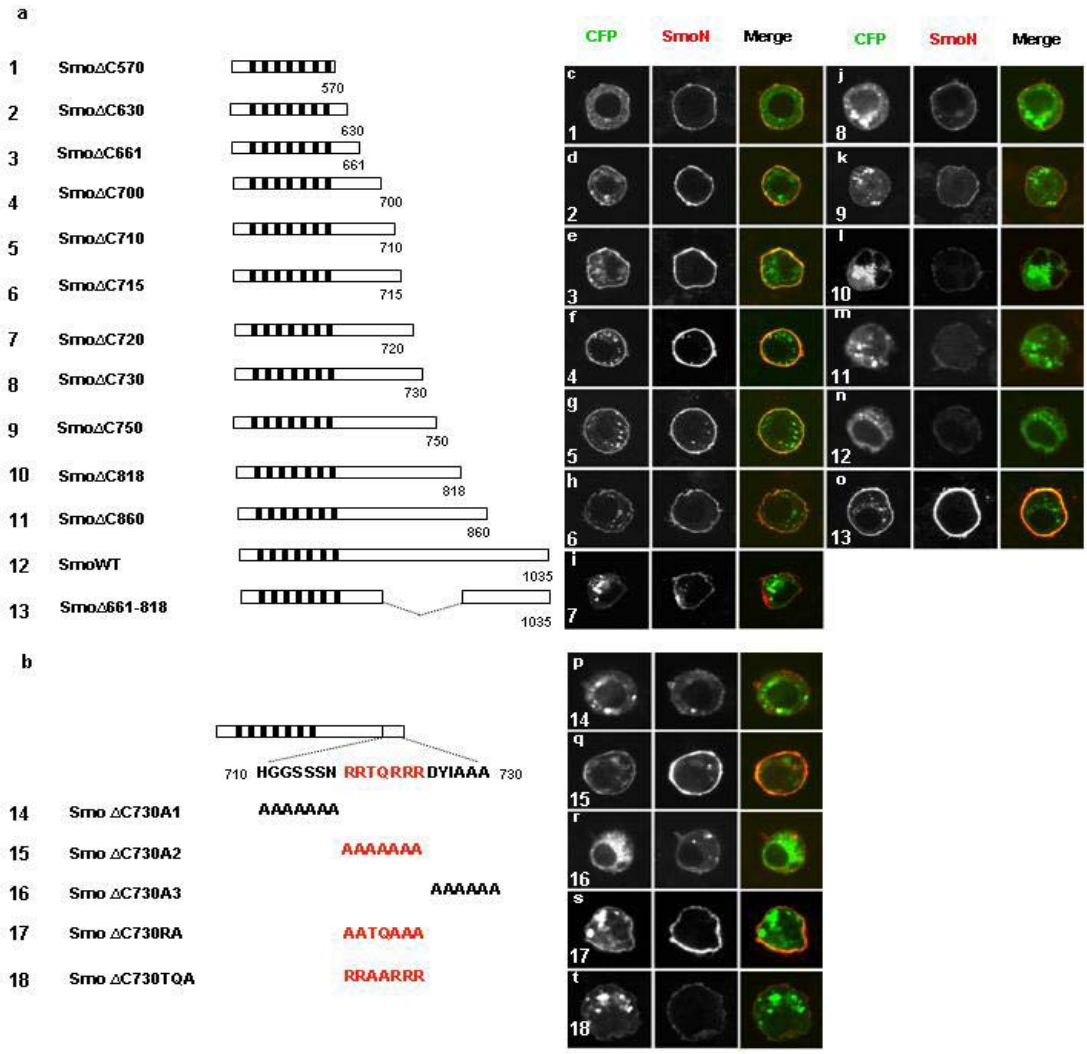



Fig. 4.1-The identification of the negative element that regulates Smo cell surface accumulation. a. CFP tagged deletion mutants. Filled boxes indicate the transmembrane domains. The amino acid residues that demarcate each deletion mutant are indicated. b. The alanine-scan mutations. SmoΔC730 with the last 20 amino acids shown underneath. A list of SmoΔC730 variants with corresponding substitutions is shown below. c-t. S2 cells were transfected with corresponding CFP tagged Smo variants as indicated. Cell surface Smo was visualized by immunostaining with anti-Smo N antibody (red channel) before cell permibilization. Deletions up to aa818 (l, m) did not change subcellular distribution dramatically. Further deletion resulted in a progressive increase of cell surface distribution of the resulting Smo variants (c-k). Smo Δ661-818 (o) has high level of cell surface accumulation. SmoΔC730A2 (q) has comparable cell surface accumulation to SmoΔC710(g). SmoΔC730A1 (p) and SmoΔC730A3 (r) showed levels of cell surface expression similar to SmoΔC730(j). The cell surface levels of SmoΔC730RA (s) but not SmoΔC730TQA (t) is similar to SmoΔC730A1.

To further characterize the negative element(s) between aa661 to aa818, more Smo C-tail deletions (Fig. 4.1a) were generated and tested by the cell surface accumulation assay. Notably, the difference of surface Smo level between SmoΔC710 (Fig. 4.1 g) and SmoΔC730 (Fig. 4.1 j) is relative dramatic compare to other adjacent deletions. To further narrow down the negative element(s) in this region, we carried out alanine scan mutagenesis in which seven or six contiguous residues were substituted into alanine at a time. Three substitutions were made to cover the region between aa710 to aa730 in the context of SmoΔC730 (Fig, 4.1b). The surface accumulation of Smo was examined for individual alanine scan mutant. The substitution from RRTQRRR to alanines (Fig. 4.1 q) but not other Ala scan mutants (Fig. 4.1 p, r) dramatically increased the cell surface expression of SmoΔC730. Since this region contains multiple arginines, we speculated that the Arg clusters may encode the negative elements. To test that, we substituted either those five Arg or the middle Thr and Gln to alanines in the context of SmoΔC730 and test their cell surface levels. As shown in Fig.4.1 s, the Arg substitution but not the TQ substitution (Fig. 4.1 t) greatly increased SmoΔC730 level, indicating that the Arg cluster prevents Smo accumulation on cell surface.

Notably, there is a graded increase of Smo cell surface accumulation when Smo is deleted from aa818 to aa661 gradually (Fig. 4.1 e-i), implying that there are multiple negative elements present in this region. Interestingly, there are several additional arginine clusters in the region between aa661 to aa818. The Arg clusters were arbitrarily named R1 to R4. With the exception of R1, which consists of $Arg_{664}$ and $Lys_{665}$, all the other Arg clusters contain two or more Arg residues. To determine whether these Arg

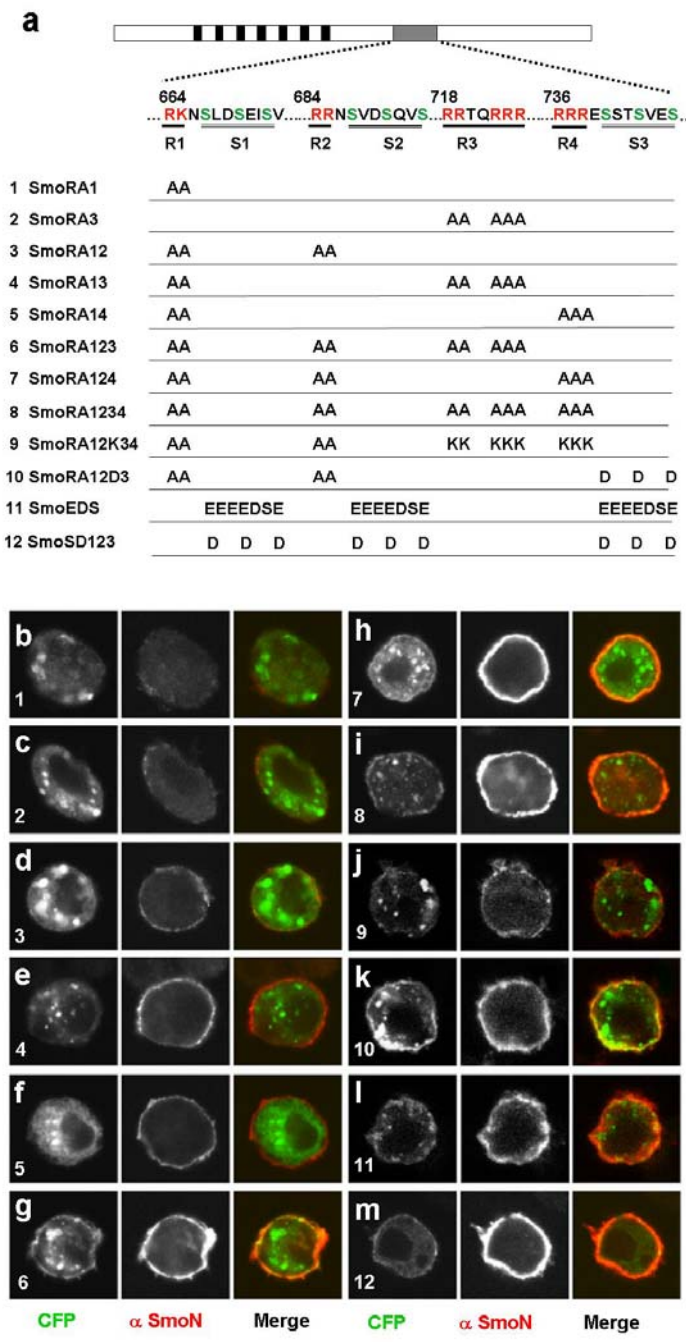



Fig. 4.2- The regulation of Smo cell surface accumulation by Arg clusters. a. Full length Smo with four Arg clusters (R1-R4, indicated by single underline) and three phosphorylation clusters (S1-S3, indicated by double underline) shown underneath. A list of Smo variants (1-12) with corresponding substitutions is shown below. b-m. S2 cells were transfected with CFP tagged Smo variants as indicated. The surface levels of corresponding Smo variants were visualized by immunostaining with anti-Smo N antibody (red channel) before cell permibilization. Smo variants with one Arg cluster changed to Ala (b, c) showed same levels of cell surface accumulation as wild type Smo.

Smo variants with two (d-f), three (g, h), or four (i) Arg clusters mutated exhibited progressive increases of cell surface accumulation. The cell surface levels of SmoRA12K34 (j) were comparable to that of SmoRA12 (d). SmoRA12D3 (k) exhibited same levels of cell surface accumulation as Smo variants with three Arg clusters mutated (g, h). The cell surface level of SmoEDS (l) was close to those of SmoSD (m) and SmoRA1234 (i).

clusters also play a role in regulating Smo cell surface expression, we introduced R to A (RA) mutations into the full-length Smo in individual or different combinations of Arg clusters (Fig. 4.2 a). Smo variants with one Arg cluster mutated (SmoRA1 and SmoRA3) (b, c) did not exhibit any significant changes in their cell surface expression levels compared with the wild type Smo (Fig. 4.1 n). However, Smo variants with two (SmoRA12, SmoRA13, and SmoRA14)(Fig. 4.1 d-f), three (SmoRA123, SmoRA124)(Fig. 4.1 g, h), or four (SmoRA1234) (Fig. 4.1 i) Arg clusters mutated exhibited progressive increases in their cell surface expression levels, that is, Smo mutants with more Arg clusters mutated showed higher levels of cell surface expression than Smo mutants with less clusters mutated. Taken together, these results demonstrate that multiple Arg clusters in the Smo C-tail act additively or cooperatively to prevent Smo cell surface accumulation.

To determine if those arginine clusters also negatively regulate Smo signalling activity, we assayed the activity of CFP-tagged wild type or Smo variants with one, two, three, and four arginine clusters substituted to alanine (Fig. 4.2 a). Each Smo construct was expressed using the *MS1096 Gal4* driver, which expresses Gal4 near-uniformly along the A/P axis in the wing pouch region. Overexpression wild type Smo-CFP induced ectopic *dpp-lacZ* expression (Fig. 4.3 a), indicative of low level of pathway activation. Smo RA single mutants (e.g. SmoRA1, SmoRA3) showed activities similar to

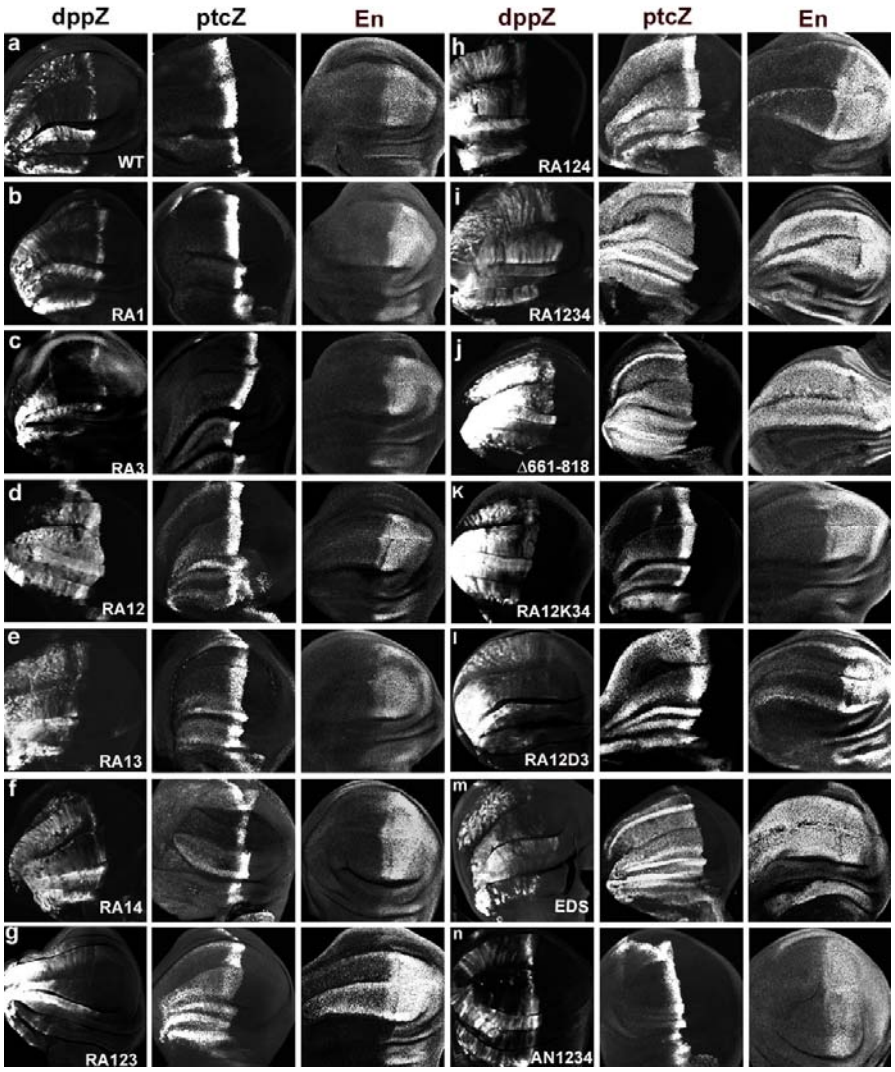



Fig. 4.3- Hh signaling activity of Smo variants. Wing discs expressing Smo variants as indicated with *MS1096* were immunostained to show *dpp-lacZ*, *ptc-lacZ* and En expression. The Smo activity is inversely correlated with the number of Arg clusters. SmoRA1 (b) and SmoRA3(c) exhibited activities similar to SmoWT (a), indicated by the ectopic expression of *dpp* but not *ptc* and *en*. SmoRA12 (d), SmoRA13 (e), and SmoRA14 (f) induced higher levels of ectopic *dpp* expression and low levels of *ptc* expression. Smo variants (g, h) with three Arg clusters mutated have even higher activity, indicated by higher levels of *ptc* expression and ectopic *en* expression. SmoRA123 (g) is more active than SmoRA124 (h), suggested by the higher levels of ectopic *en* expression. SmoRA1234 (i) exhibited highest pathway activity indicated by high levels of *ptc* and *en* expression. The activity of SmoΔ661-818 (j) and SmoEDS (m) is similar to SmoRA1234. The activity of SmoRA12K34 (k) is close to SmoRA12 (d). SmoRA12D3 (l) exhibited activity similar to SmoRA124 (h). The activity of SmoAN1234 is similar to that of SmoWT.

the wild type SmoCFP (Fig. 4.3 b, c). Smo RA double mutants (e.g. SmoRA12, SmoRA13, SmoRA14) induced more dramatic *dpp-lacZ* expression in the anterior compartment and begin to induce *ptc-lacZ* expression (Fig. 4.3 d-f). In contrast, Smo RA triple mutants (SmoRA123, SmoRA124) led to high levels of *ptc-lacZ* expression and began to induce *en* expression (Fig. 4.3 g, h). Strikingly, overexpression of Smo RA quadruple mutant (SmoRA1234) resulted in highest pathway activity, as indicated by the full-blown ectopic activation of *ptc* and *en* (Fig. 4.3 i). These results indicate that Arg clusters negatively regulate Smo activity and Smo activity is inversely correlated with the number of Arg clusters.

Phosphorylation regulates Smo activity by neutralizing the positive charges carried by the Arginine clusters.

The graded activity changes of the RA mutations remind us the activity changes caused by differential phosphorylation of SmoC-tail. The similar effects caused by increasing phosphorylation levels and decreasing the number of functional Arg clusters imply that phosphorylation regulates Smo by antagonizing the Arg clusters. Strickingly, SmoΔ661-818 which lacks both Arg and phosphorylation clusters was highly accumulated on cell surface and ectopically activates high threshold Hh responsive genes such as *ptc* and *en* when overexpressed in wing imaginal discs (Fig. 4.1 o and Fig. 4.3 j). These results suggest that phosphorylation promotes Smo cell surface accumulation and activity by counteracting the negative effect of the arginine clusters. It is striking to note that the Arg clusters are situated adjacent to the PKA/CKI phosphorylation clusters. In fact, R1, R2, and R4 are part of the PKA phosphorylation consensus sequence, R/KRXS. The close association of the Arg clusters and phosphorylation sites may allow tight

control of Smo activity by phosphorylation as phosphorylation at individual clusters only neutralize the negative influence of the adjacent Arg cluster(s). To further test this, we constructed SmoRA12D3 in which R1 and R2 were mutated to Ala whereas the third phosphorylation cluster was mutated to Asp (Fig. 4.2 a). As expected, SmoRA12D3 exhibited higher levels of cell surface expression and signaling activity than SmoRA12 (Fig. 4.2 k, Fig. 4.3 l). In fact, the activity of SmoRA12D3 was similar to that of SmoRA124 (Fig. 4.2 h) in which R1, R2, and R4 were mutated. Thus, SD mutation in the third phosphorylation cluster increased Smo activity in a fashion similar to RA mutation in the adjacent Arg cluster.

Then we want to know how phosphorylation antagonizes the negative effects of the Arg clusters. Since the arginine clusters carry positive charges while the phosphorylation brings in negative charges, we speculated that phosphorylation may antagonize Arg clusters by neutralizing their positive charges. To test that, we made a Smo mutant with the first two arginine clusters mutated to alanine and last two clusters mutated to lysine (SmoRA12K34)(Fig. 4.2a) and tested its activity. We knew that Smo RA1234 has high activity while SmoRA12 only has mild activity. If the positive charges are important, we expect to see that the activity of SmoRA12K34 will be more close to that of SmoRA12. While, if the Arg itself is important, the activity of RA12K34 will be more close to SmoRA1234. Overexpression of SmoRA12K34 showed mild activity just like SmoRA12 (Fig. 4.3 k), suggesting Lys could mimic Arg to inhibit Smo activity. These data indicating the positive charges carried by Arg clusters are critical to inhibit Smo activity.

To further test whether phosphorylation is to neutralize the positive charges carried by Arg residues, we made a Smo mutant with all three phosphorylation clusters

changed to stretches of acidic amino acid (SmoEDS)(Fig. 4.2 a) , and tested its cell surface expression level and activity. SmoEDS accumulated on cell surface (Fig. 4.2 l) and ectopically activated high threshold Hh responsive genes such as *ptc* and *en* when overexpressed in wing imaginal discs (Fig. 4.3 m), indicating that the acidic amino acid stretches could mimic phosphorylation and promote Smo cell surface accumulation and activity possibly by neutralizing the positive charges carried by the Arg clusters.

Arg clusters maintain Smo C-tail form an inactive "closed" conformation by interacting with acidic clusters.

Although the Arg clusters on Smo C-tail appear to be important in regulating Smo activity, the mechanism is still elusive. In a parallel study, we have obtained evidence that phosphorylation triggers a conformational switch in Smo essential for its activation (Zhao, Y, unpublished data). In the absence of Hh, Smo appears to adopt a closed inactive conformation in which its C-terminus (CT) is in close proximity to its third intracellular loop (L3). Hh-induced phosphorylation at PKA and CKI sites triggers a conformational change in which the Smo CT moves away from its L3 and closer to the CT of another Smo within a dimer. One mechanism by which the Arg clusters inhibit Smo activity is to keep Smo in the close inactive conformation. To test this, we examined whether mutating the Arg clusters causes conformational changes similar to phosphorylation at PKA/CKI sites. To measure the distance between Smo CT and L3, we employed fluorescent resonance energy transfer (FRET) analysis using a Smo biosensor, Smo-CFP$^{L3}$YFP$^{C}$, in which the Smo is doubly tagged with CFP inserted in the L3 and YFP fused to the CT. When expressed in S2 cells, the FRET efficiency of Smo-

$CFP^{L3}YFP^{C}$ was ~13% in the absence of Hh treatment; however, the FRET dropped to ~4.3% in response to Hh treatment. The FRET efficiency also dropped to low levels by the phosphorylation-mimicking mutations (Zhao, Y., unpublished data). Interestingly, mutating the Arg clusters or deleting the region between aa661-818 in Smo-$CFP^{L3}YFP^{C}$ resulted in constitutively low levels FRET, as was the case for the SD mutation (Zhao, Y, unpublished data), indicating that the Arg clusters are important for keeping the Smo CT and L3 in close proximity and this effect is abrogated by Hh-induced phosphorylation at PKA/CKI sites.

We went on to ask how the Arg motifs maintain Smo in the closed inactive conformation. One possibility is that the closed conformation is maintained by intra-molecular interactions between a C-terminal region and the region containing multiple Arg clusters. To test this, we carried out GST pull down experiments. We constructed a GST fusion protein (GST-Smo656-755) that contains the Smo sequence between aa656 to aa755. This region includes the Arg clusters R1-R4 and the PKA/CKI phosphorylation clusters S1-S3, and is referred to hereafter as SAID for Smo auto-inhibitory domain. GST-Smo656-755 was incubated with a series of C-terminal Smo fragments either expressed in S2 cells or produced by *in vitro* translation. From this assay, we identified a minimal SAID interacting fragment (NT860) that contains the C-terminal region between aa 860-1,035 (Fig. 4.4 a). The interaction between NT860 and SAID was inhibited by phosphorylating GST-Smo656-755 with PKA and CKI (Fig. 4.4 b). In addition, this interaction was abolished by either the RA or SD mutation but not by the SA mutation (Fig. 4.4 b). Interestingly, we observed a gradual decrease in the binding affinity when increasing number of Arg or phosphorylation clusters were altered (Fig. 4.4 c). The

interaction between NT860 and SAID is likely to reflect an intra-molecular rather than an intermolecular interaction as wild type Smo C-tail failed to interact with each other in the yeast (Zhao, Y, unpublished data).

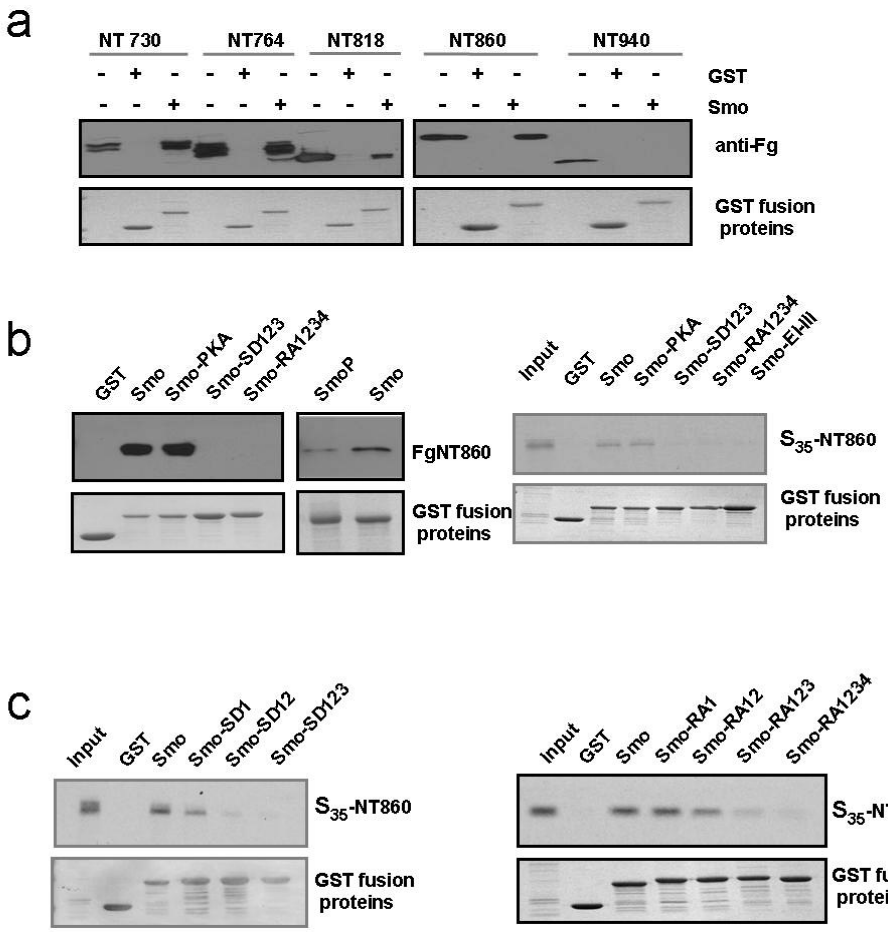



Fig. 4.4-Arg clusters mediate Smo C-tail intramolecular interaction. Cell extracts derived from S2 cells transfected with indicated Smo N-terminal deletions (a, b left panel) or reticulocyte lysates containing corresponding $S_{35}$- labeled proteins (b right panel, c, and e) were incubated with indicated GST or GST fusion proteins immobilized on glutathione beads for 1 hour at 4°C. The beads were washed five times with lysis buffer, followed by Western blotting with antibodies as indicated (upper panels of a, b left panel) or running SDS-PAGE and performing autoradiography (upper panels of b right panel, c). The total GST or GST fusion protein inputs (lower panels of a, b, c,) were showed by Commassie Blue staining following seperating on SDS-PAGE. In panel a, 1/25 cell lysates were run side by side as the inputs of Smo N-terminal deletions. In panel b (right panel) and c, 1/2 *in vitro* translated samples were loaded as inputs. In panel b, the *in vitro* phosphorylated Smo is indicated as SmoP.

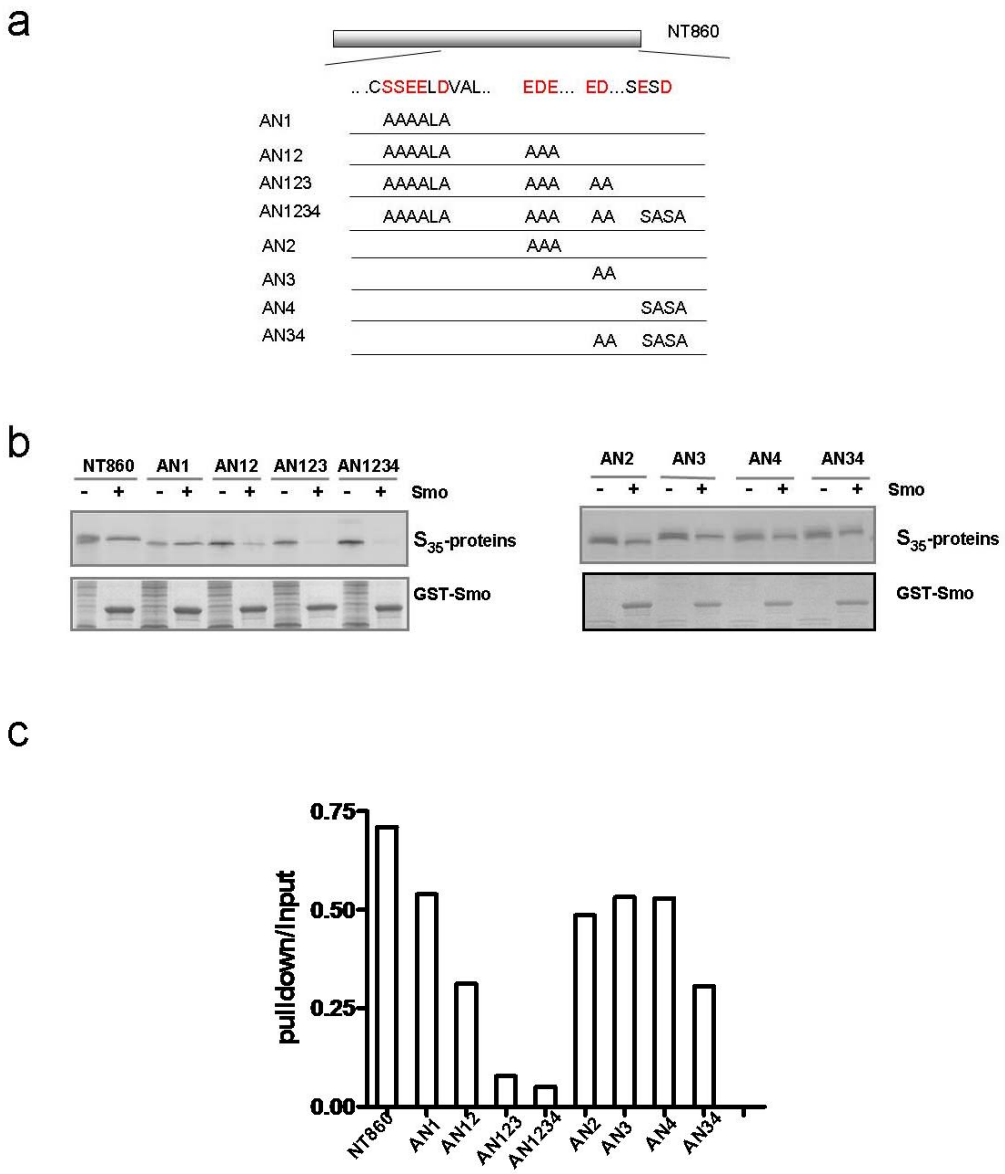



Fig. 4.5- Arg clusters interact with acidic clusters in Smo C-tail. a. Smo N-terminal truncation NT860 was shown with four acidic clusters indicated underneath. A list of SmoNT860AN mutations with corresponding substitutions is shown below. b. Reticulocyte lysates containing corresponding $S_{35}$- labeled proteins were incubated with indicated GST-Smo fusion protein immobilized on glutathione beads for 1 hour at 4°C. The beads were washed five times with lysis buffer, followed by running SDS-PAGE and performing autoradiography (upper panel). The total GST-Smo fusion protein inputs (lower panel) were showed by Commassie Blue staining following running SDS-PAGE. 1/2 *in vitro* translated samples were loaded as inputs. c. the quantification of panel b. The pull down efficiency was determined by the intensity of pull-downed protein divided by the intensity of the input. The intensity of the bands on the image showed in panel b was measured by Image J.

Since the positively-charged Arg clusters are essential for NT860/SAID interaction, we examined the possibility that NT-860/SAID association could be mediated by electrostatic interactions. Indeed, we identified four acidic clusters in the C-terminal half of NT860 (Fig. 4.5 a). To determine whether these acidic clusters are important for NT860/SAID interaction, we mutated them to Ala either individually or in different combinations. Whereas mutating any individual acidic clusters did not significantly affect the binding of NT860 to GST-Smo656-755, mutating two or more acidic clusters simultaneously reduced or abolished the binding (Fig. 4.5 b). Interestingly, we observed a gradual decrease in the binding affinity when more acidic clusters were mutated, as was the case when more Arg clusters were mutated, suggesting that NT-860/SAID association is mediated by electrostatic interactions between multiple Arg in SAID and multiple acidic clusters near Smo CT.

Thus, the intra-molecular electric static interactions may result in a folding back of the Smo C-terminal region to form a closed conformation. Consistent with this notion, mutating the acidic clusters in Smo-CFP$^{L3}$YFP$^{C}$ decreased the FRET efficiency, indicative of increased distance between the CT and L3 (Zhao, Y, unpublished data). However, mutating the acidic clusters did not render the resulting Smo variant (SmoAN1234) constitutively active. Instead, SmoAN1234 exhibited wild type Smo activity (Fig. 4.3 n). Consistent with its lack of constitutive activity, SmoAN1234 exhibited little if any cell surface expression in the absence of Hh (data not shown).

## Discussion

Taken together, we propose a model that Arg clusters in SmoCT prevent Smo cell surface accumulation and keep SmoCT in a "closed" inactivive conformation. Most importantly, we found phosphorylation antagonizes the negative effects of Arg clusters and multiple Arg clusters endow Smo the ability to respond to different levels of Hh through differential phosphorylation.

Arginine clusters negatively regulate Smo cell surface accumulation

In this study, we found that multiple arginine clusters negatively regulate Smo cell surface expression. The Arg clusters may function as reverse trafficking signals that promote Smo endocytosis and subseqent degradation in lysosomes whereas phosphorylated acidic clusters could provide forward trafficking signals to promote recycling. It has been shown that the di-basic motifs such as RR, RXR and KR could serve as ER retention signals by interacting with β-COP, a component of the coat protein complex I (COPI). Once the protein is phosphorylated in the same region, β-COP was dissociated from the di-basic motif and 14-3-3 was recruited, which results in the forward trafficking of the protein (O'Kelly et al., 2002). Strikingly, both β-COP and δ-COP were identified as negative regulator of Hh signaling pathway (Lum et al., 2003a), which raises a possibility that they may interact with the arginine motif of Smo and prevent Smo cell surface accumulation by trapping Smo in ER. However, we found that mutating the arginine motifs in Smo does not affect the interaction between Smo and β-COP (data not shown). Furthermore, the majority of Smo is localized in the lysosomes instead of ER in absence of Hh (Nakano et al., 2004), suggesting that β-COP is not likely to be the trans-regulator that binds to the arginine clusters and inhibits Smo cell surface expression.

Although our data indicate that phosphorylation at PKA and CKI sites becomes dispensable when the Arg clusters are removed, we noticed the SD mutations behaved differently comparing to the RA mutations in certain circumstances. We found that fusion of a Smo fragment from aa661 to aa818 at the C-terminal to Fz resulted in most of the chimera protein accumulated in cytoplasm instead of cell surface, whereas fusion of the same fragment with S to D mutations restored cell surface expression of the chimera protein. However, fusion of the same fragment with R to A mutations only partially restored cell surface expression of the chimera protein (Fig. 4.6). These data suggest that the phosphorylation of Smo at PKA and CKI sites not only neutralizes the negative effects of the arginine cluster, but also provides additional signals to promote Smo cell surface accumulation. It is also possible that there are additional negative signals beside Arg clusters in Smo C-tail.

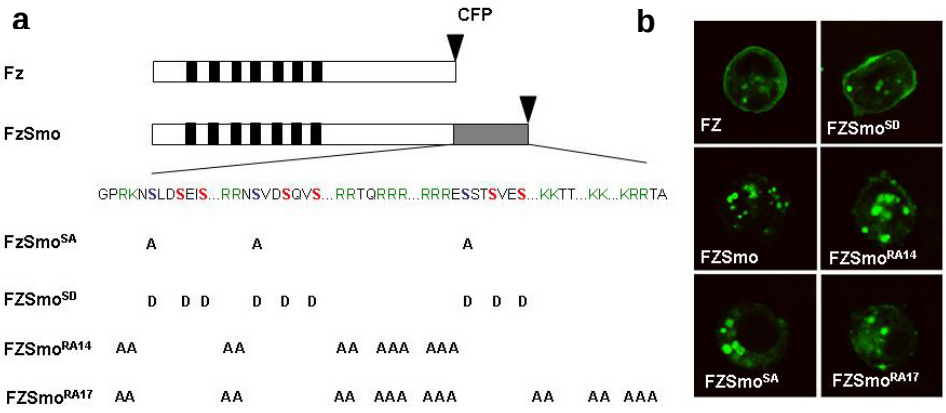



Fig. 4.6- Phosphorylation on SmoCT provides additional signals to promote Smo cell surface accumulation. a. CFP tagged Fz and FzSmo chimera was shown. The black boxes indicate the transmembrane domain. The gray box and triangles indicate Smo fragment aa661-aa818 and CFP, respectively. The phosphorylation clusters (blue and red letters) and Arg clusters (green letters) in Smo were shown underneath. A list of FzSmo mutants has indicated substitutions are shown below. b. S2 cells transfected with indicated expressing constructs were fixed and observed by confocol microscope. The majority of Fz is localized on cell membrane. Fusion of wild type or S to A mutant form of Smo aa661-818 reduced FzSmo cell surface expression. Fusion of corresponding Smo

fragment with SD mutations restored FzSmo cell surface accumulation. FzSmo$^{RA14}$ and FzSmo$^{RA17}$ only partially restored cell surface distribution.

Future study will investigate how Smo trafficking is regulated by multiple Arg motifs and phosphorylation.

Multiple arginine clusters negatively regulate Smo activity.

The Arg clusters negatively regulate Smo at several levels that may all contribute to reducing Smo activity. First, they keep Smo in a closed inactive conformation in which the C-terminal region of Smo folds back and interacts with SAID. Second, the Arg clusters prevent Smo cell surface accumulation thus further limit the overall pathway activity. The recent study carried in our lab also indicated that the Arg clusters prevented close proximity of Smo C-tails, which is essential for Smo activation (Zhao, Y, unpublished data). All these layers of negative regulation can be counteracted by PKA and CKI mediated phosphorylation of Smo C-tail.

Our data suggest that the Arg clusters exert their negative influence through their positive charges and phosphorylation antagonizes the Arg clusters by bringing in negative charges. The positive charges of the Arg clusters keep Smo in the closed conformation through electrostatic interactions with the negative charged of the acidic clusters near the Smo C-terminus. The positive charges could also prevent close proximity of Smo C-tails through electric repulsion (Zhao, Y, unpublished data).

An important feature of SAID is that it contains multiple regulatory modules each of which consists of an Arg cluster linked to a phosphorylation cluster. The pairing of positive and negative regulatory elements may offer more precise regulation as phosphorylation at a given cluster only neutralizes adjacent negative element(s), leading to an incremental change in Smo activity. Increasing phosphorylation gradually

neutralizes the negative effect by multiple Arg clusters, leading to a progressive increase in Smo cell surface expression and activity. Thus, employing multiple such regulatory modules allows Smo activity to be fine tuned by differential phosphorylation in response to different levels of Hh (Fig. 4.7).

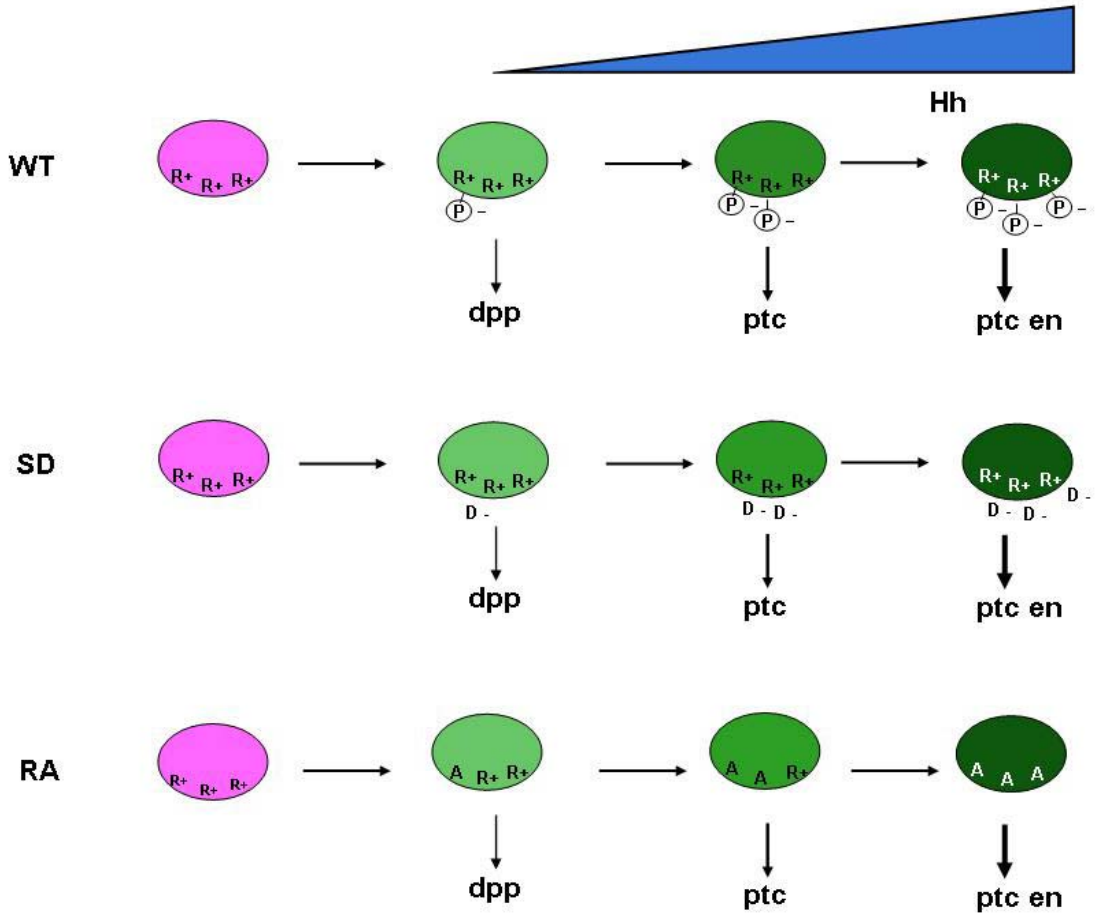



Fig. 4.7- The regulation of Smo activity in response to gradient Hh concentration. With the increasing of Hh levels, the levels of Smo phosphorylation are also elevated. Increasing phosphorylation gradually neutralizes the negative effects by multiple Arg cluster, leading to progressive increase in Smo activity. The SD mutations and RA mutations mimic the differential phosphorylation and progressive neutralizing the negative effects, respectively. Therefore different mutation forms of Smo have different activities.

The divergence of Smo regulation between invertebrate and vertebrate

As the sole Hh signal transducer across cell membrane, Smo is regulated surprisingly differently between insects and vertebrates. First of all, the insect Smo tail is longer than the vertebrate one. Secondly, the phosphorylation clusters are absent from the vertebrate Smo C-tail, although they are very important for Smo activity in *Drosophila*. Here we identified multiple arginine clusters which negatively regulate Smo cell surface expression and activity in *Drosophila*. Intriguingly, there are several arginine clusters R1, R2, R3, and R4 in vertebrate Smo C tail (Fig. 4.8). We asked whether these arginine clusters also negatively regulate vertebrate Smo activity. We gradually substituted the arginine clusters to alanines to make single (RA4), double (RA34, RA12), triple (RA234), and quadruple (RA1234) mutants of mouse Smo and test the activity of these mutants by using Gli-luciferase assay in NIH3T3 cells. As shown in Fig. 4.8, RA12 and RA1234 exhibited lower activity than wild type, while the activity of RA234, RA34 and RA4 is comparable to wild type mSmo, suggesting R1 cluster may essential for mSmo activity and other Arg clusters is dispensable for mSmo activity. These data implying that the arginine clusters play different roles in different organisms and diverse regulation strategies are used during the evolution. More vigorous experiment should be done to further confirm these *in vitro* assay data.

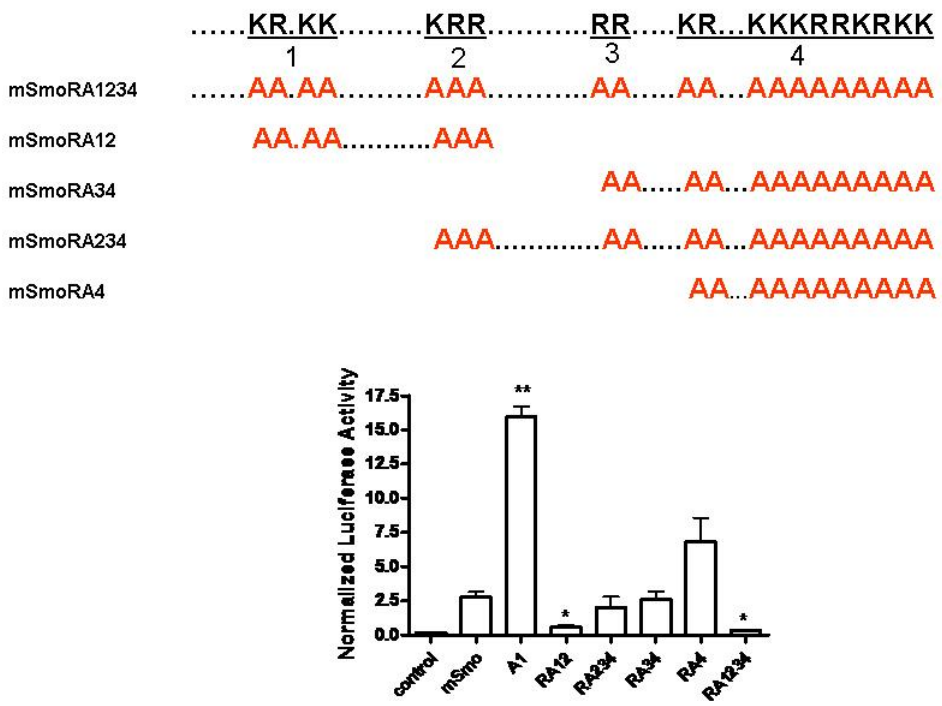



Fig. 4.8- The Arg clusters in mouse Smo (mSmo) play different roles. The Arg clusters in mSmo were shown. A list of RA mutations with indicated Arg clusters substitutions is shown below. NIH3T3 cells were transfected with indicated expressing constructs to in conjugation with Gli-luciferase and TK-Renila expression vector. One day after transfection, cells underwent serum starvation (0.5% FBS) for additional two days. Then the luciferase assays were performed. The luciferase activity was normalized by lusiferase activity divided by Renila activity. SmoA1 (A1), a mutant form of Smo with constitutive high activity, was used as a positive control. A student t test was performed between the wild type Smo and the individual mutants. The asterisks indicating the differences between wild type Smo and the mutant forms of Smo are statistic significant. (** indicating $p<0.01$, * indicating $p<0.05$).

## Methods

Plasmid constructions and transgenes

Smo C terminal deletions constructs were generated by a PCR based approach followed by subcloning into pUASCFP vector. The CFP was fused to the C-terminal of the Smo variants. The alanine scan constructs and Smo variants with substitutions at the phosphorylation clusters, arginine clusters, and acidic clusters were generated by site-directed mutagenesis. To generate pGEX-Smo variants (pGEX-Smo, -Smo-PKA, -SmoSD1, -SmoSD12, -SmoSD123, -SmoRA1, -SmoRA12, -SmoRA123, -SmoRA1234, -SmoEDS), *smo* cDNA fragments encoding Smo aa656-755 and corresponding variants with phosphorylation site or Arg cluster substitutions were amplified by PCR and inserted between NotI and EcoRI sites on the pGEX4T-2 vector. To construct Flag tagged Smo N terminal deletions (pUASSmo-NT730, -NT764, -NT818, -NT860, -NT940), *smo* cDNA encoding fragments from indicated start amino acid residue to aa1,035 were amplified and subcloned to PUAST-Flag. Various pcSmoNT860 AN mutants were generated by using quick change site directed mutagenesis kit (stratagene) and using pcSmoNT860 as template. The mutant sites were verified by sequencing. Fly transformants were generated by standard P-element mediated transformation. Multiple independent transgenic lines were tested for each construct. *MS1096*, *dppZ*, and *ptcZ* were described before.

Cell culture, immunoblot, GST pull down, immunostaining and in vitro phosphorylation.

Cell culture, transfection and Hh-condition medium treatment were carried out as described in chapter two. For *in vitro* GST-Smo phosphorylation, GST fusion protein was absorbed on glutathione beads (Amersham) and incubated with recombinant PKAc

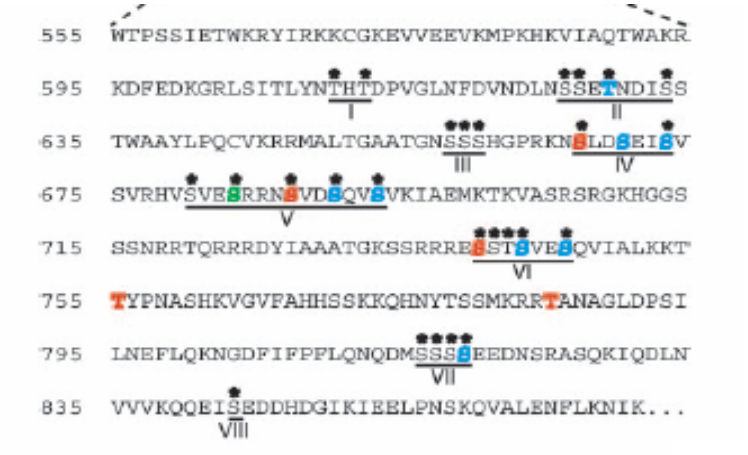
and CKI δ (NEB) at 30°C for 2 hours after thoroughly wash. For the GST fusion protein pull-down assay, GST fusion proteins absorbed on glutathione beads were washed with ice-cold PBS containing 1% NP40 three times. S2 cell lysate with FgSmoNT860 over expression or in vitro translated $^{35}$S-labeled either wild type SmoNT860 or corresponding mutants were then added and the mixtures were incubated at 4ºC for 1 hour with occasional mixing. Proteins bound to the beads were washed with PBS plus 1% NP40 five times before separation on SDS-PAGE. The in vitro translation was carried out by using the TNT coupled reticulocyte lysate system (Promega). We performed western blotting analysis as described. Cell-surface staining and immunostaining of imaginal discs were performed as described in chapter three. Antibodies used in this study: mouse anti-En (DSHB), rabbit anti- βGal (Cappel), mouse anti-Flag (Sigma), and mouse anti-SmoN (DSHB).

# CHAPTER FIVE

## Gprk2 is required for Smo to transduce high levels of Hh signaling activity

### Introduction

In our previous study, we identified PKA and CKI phosphorylation sites organized in three clusters that are essential for Smo activity. In an independent study, Zhang et al. (Zhang et al., 2004) identified another seventeen sites in Smo C-tail that are phosphorylated *in vivo*, in addition to the nine sites we identified (Fig 5.1). Consistent with this finding, we found that FgSmo$^{-PKA123}$ still exhibited low levels of phosphorylation *in vivo* (chapter three). The kinases responsible for phosphorylating those sites wait to be identified and the function of these phosphorylation events remains unknown.




Zhang, C *et al*., 2004

Fig. 5.1- The Hh induced phosphorylation sites on Smo C-tail (from Zhang, C *et al*. 2004). The red S/T indicats potential PKA site. The blue S/T indicated potential CKI site, the green S indicated the potential GSK3 site. The letters with underline are shown the eight phosphorylation clusters in Smo C-tail. The S and T with asterisk above indicate the phosphorylation sites identified by mass spectrometery.

Most of the phosphorylation sites in *Drosophila* Smo are not conserved in vertebrate Smo. However, recent studies (Chen et al., 2004; Wilbanks et al., 2004) showed that vertebrate Smo could be phosphorylated by G protein coupled receptor kinase 2 (GRK2) and recruited β-Arrestin 2 to plasma membrane. Both the phosphorylation of Smo and β-Arrestin 2 recruitment are inhibited by Ptc and cyclopamine and promoted by Shh and Smo agonist.

In an effort to identify new Hh signaling pathway components, our lab has carried out genetic screen. A deficiency line lacking a *Drosophila* GRK gene, *gprk2*, enhanced *hh* mutant like phenotype caused by expressing a dominant negative form of Smo. Interestingly, *gprk2* gene expression is up-regulated at the A/P boundary (Schneider and Spradling, 1997), indicating that the expression of this gene may be regulated by Hh signaling.

Although GRK2 appears to be required for vertebrate Smo phosphorylation, GRK2 sites have not been defined. It is also unclear if GRK2 directly regulates Smo by phosphorylation. *Drosophila* Smo is phosphorylated in response to Hh and multiple phosphorylation events are required for Smo activity. The identified phosphorylation sites in *Drosophila* Smo are phosphorylated by PKA and CKI. If the functions of phosphorylation and β-Arrestin2 are conserved between *Drosophila* and vertebrates, PKA and CKI phosphorylation sites may serve the same purpose and recruit Arrestin as has been proposed for GRK2 in regulating vertebrate Smo. In addition to the PKA and CKI sites, there are many uncharacterized phosphorylation sites on *Drosophila* Smo C-tail. As showed in Fig 5.1, those phosphorylation sites form eight clusters and most are in the vicinity of the acidic residues, as found for GRK sites in the β2-adrenergic receptor. It

is possible that some of the uncharacterized phosphorylation sites in *Drosophila* Smo are Gprk2 sites.

In response to Hh, *Drosophila* Smo is accumulated on the cell surface. The phosphorylation at PKA and CKI sites appears to promote Smo cell surface expression by either inhibiting Smo endocytosis or promoting Smo recycling (Jia et al., 2004). However, until now, it is not clear how Smo trafficking is regulated. In vertebrate cell lines, Smo undergoes GRK2 and β-Arrestin2 dependent internalization upon the stimulation with Smo agonist (Chen et al., 2004). However, the biological significance of Smo internalization by β-Arrestin2 is not clear. The interaction with β-Arrestin2 may facilitate vertebrate Smo traffick to unique subcellular component for signaling, such as primary cilia, which has been implicated in Shh signaling and contains almost all the known Hh pathway components. In *Drosophila*, whether GRK and Arrestin play similar role awaits to be tested.

In this study, we found that Gprk2 is essential for high threshold Hh signaling activity. Surprisingly, Gprk2 seems to have kinase activity independent role in Hh signaling pathway. Furthermore, we also identify a new CKI phosphorylation cluster in Smo C-tail. We provided evidence that this cluster is critical for Smo subcellular localization and activity.

## Results

Gprk2 is essential for high threshold Hh signaling activity.

To determine if Gprk2 is essential for Hh signaling activity, we generate somatic clones in wing imaginal discs with a *gprk2* P element insertion allele, $gprk2^{06936}$. As showed in Fig. 5.2, in posterior compartment clones, Smo levels remained the same (Fig. 5.2 b); however, Smo levels increased in A-compartment *gprk2* clones close to the A/P boundary (Fig. 5.2 a, c). Neverless, Ci was not stabilized in these clones and no ectopic expression of downstream genes such as *dpp* or *ptc* was observed (Fig. 5.2 a, c, and data not shown). In clones butting the A/P border, there was no dramatic change of the Smo levels, however, *en* expression was significantly reduced and the corresponding "low Ci155" region exhibited elevated levels of Ci (Fig. 5.2 d), suggesting that high threshold Hh responces are compromised. In anterior clones far away from A/P boundary, there was only slight increase of Smo level (Fig. 5.2 c). Since $gprk2^{06936}$ is not a null allele, we generate another Gprk2 mutant allele D15 by non-precise excision of the P-elements. The phenotype of D15 was similar to but more severe than that of $gprk2^{06936}$ (data not shown).

Since the $gprk2^{06936}$ allele might affect a neighboring gene CG11337, to confirm that the mutant phenotype is due to loss of Gprk2, we employed an inheritable RNAi technique to inactivate Gprk2 (Kalidas and Smith, 2002). Consistent with the results from $gprk2^{06936}$ mutant clones, expressing a Gprk2 RNAi construct in wing imaginal discs with *ApGal4* led to Smo elevation in A-compartment cells near the A/P border and diminished *en* expression in the dorsal part of wing discs (Fig. 5.3). However, *ptc-lacZ* expression was not affected. These data indicate that Gprk2 is only essential for the expressing of

high threshold Hh target gene such as *en* and it is likely to regulate Hh signaling pathway by regulating Smo.

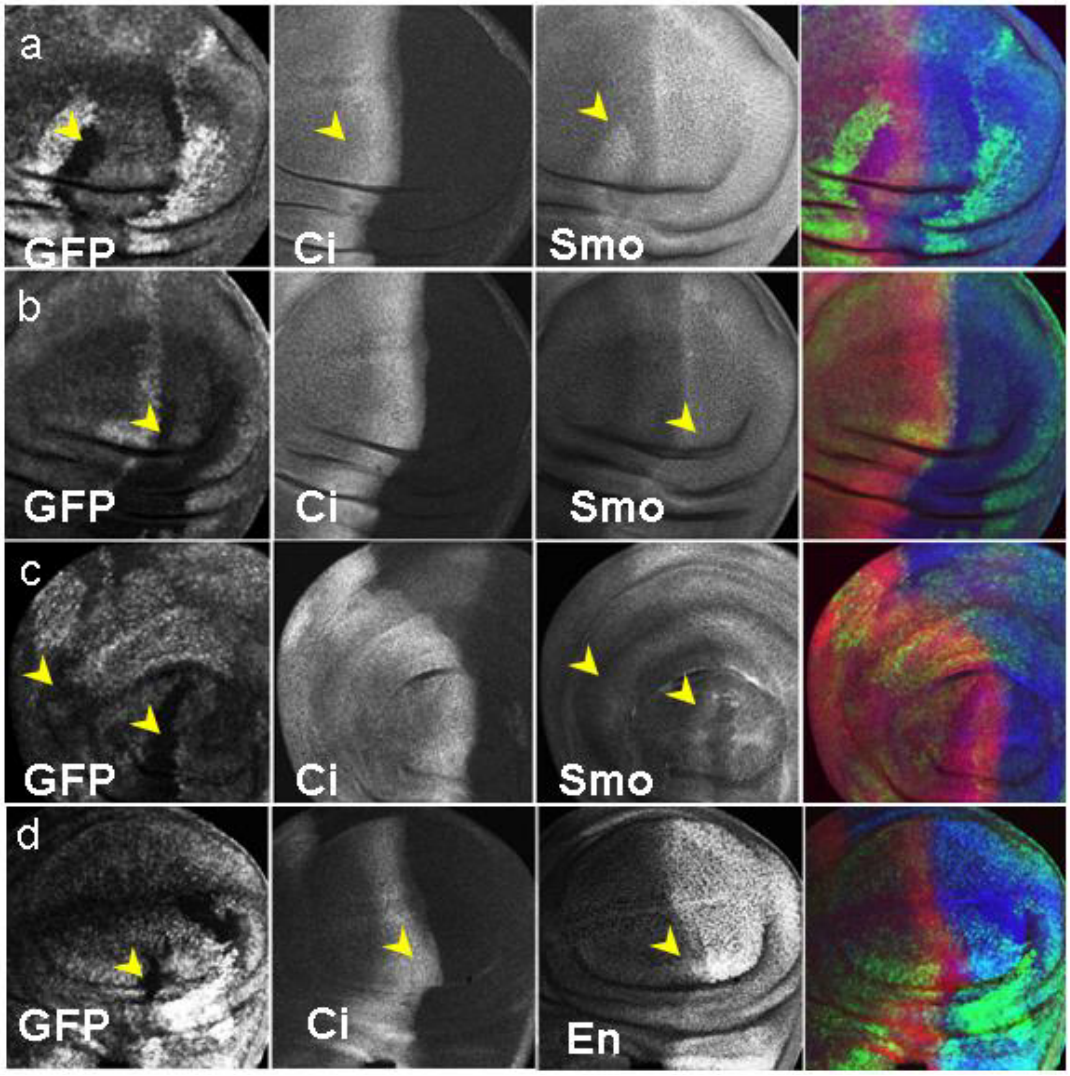



Fig. 5.2 – Gprk2 is essential for Hh signaling activity. Wing discs carry *grpk2* clones (marked by lack GFP staining) were stained to show the expression of GFP, Ci, Smo (a-c) and En (d). In the clones near A/P boundary, the level of Smo is elevated (a,c), but Ci processing is not blocked. In the posterior clones (b), the level of Smo remains same. In the clones on the A/P boundary, En expression is reduced, at the same time Ci level is increased (d).In the clones far away from A/P boundary, the increase of Smo level is not dramatic (c).

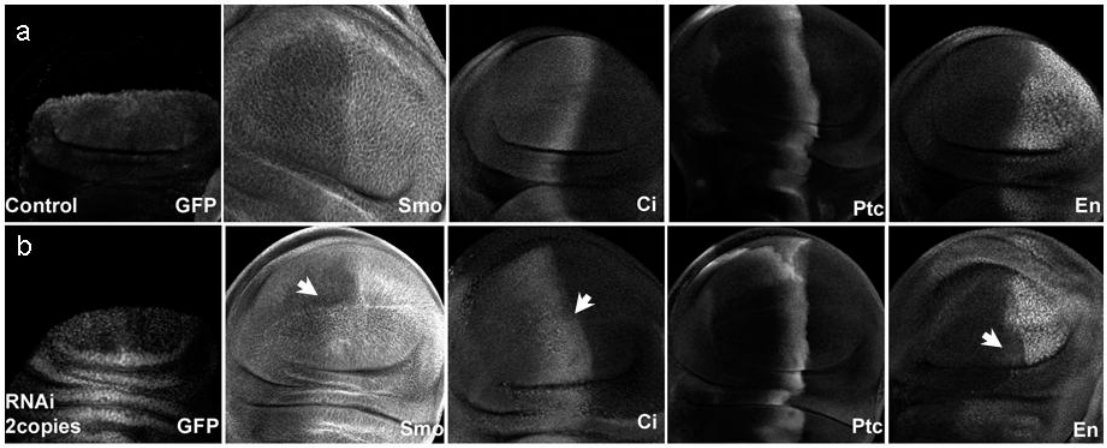



Fig. 5.3- Gprk2 RNAi shows phenotype similar to Gprk2 mutants. Wing disc expressing two copies of *UAS-Gprk2-RNAi* with *ApGal4* were stained (b) to show the expression of Smo, Ci, ptc-lacZ and En. The *ApGal4* expression region is marked by GFP staining. *gprk2* knock down results in expansion of the high level Smo expression domain, stabilizing Ci and reducing of En expression at the A/P boundary (indicated by arrows).

To test whether Gprk2 could associate with Smo, we overexpressed Flag-tagged Gprk2 (Fg-Gprk2) and Myc-tagged Smo (Myc-Smo) in S2 cells and immunoprecipited MycSmo, followed by Western blotting to detect Fg-Gprk2. As shown in Fig.5.4 a, Fg-Gprk2 was pulled down by MycSmo. To further determine which part of Smo is required for Smo-Gprk2 interaction, we transfected S2 cell with MycSmoΔC and MycSmoCT, followed by immunoprecipitation and western blot. As shown in Fig.5.4 a, SmoCT but not SmoΔC interacted with Gprk2. Then we went on to further test whether PKA and CKI mediated Smo phosphorylation affect Smo-Gprk2 interactions. Myc-tagged Smo variants (MycSmo, MycSmo$^{-PKA123}$, MycSmo$^{SD123}$) were transfected to S2 cells together with Fg-Gprk2. Cell lysate was immunoprecipited with anti-Myc antibody and the IP products were analyzed by western blotting. There was no dramatic difference in the amount of FgGprk2 pulled down by differnt Smo variants (Fig. 5.4 b), indicating that

phosphorylation at PKA and CKI sites between aa661 to aa818 does not regulate Gprk2-Smo interactions.

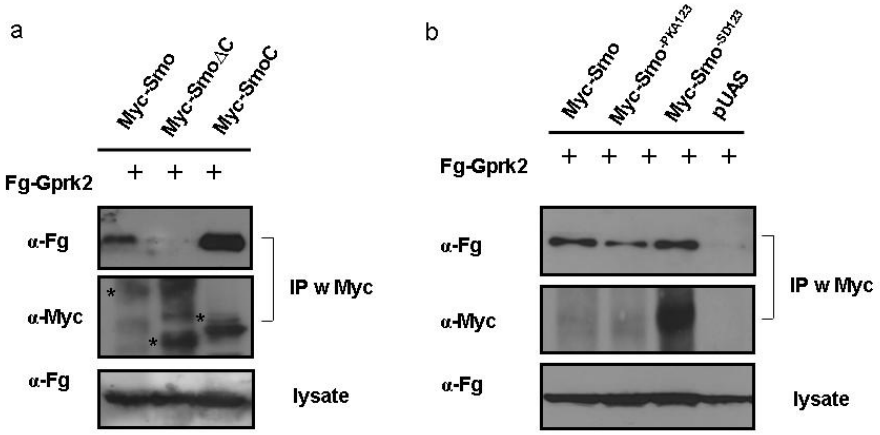



Fig. 5.4- Smo interacts with Fg-Gprk2 through its C-terminal tail. S2 cells were transfected with Myc-Smo (a), Myc-SmoΔC(a), Myc-SmoC(a) or various phosphorylation mutants (b) together with Fg-Gprk2. Cell extracts were immunoprecipitated by anti-Myc antibody, followed by western blotting with the antibodies indicated. 5% whole cell lysates were also analyzed by immunoblotting with anti-Fg antibody. Myc-Smo and Myc-SmoC but not Myc-SmoΔC pulled down Gprk2. Both $Smo^{-PKA}$ and $Smo^{SD}$ pulled down Gprk2. The different amount of Gprk2 pulled down by various Smo mutants may reflect the difference of the expression level of Smo mutants. The asterisks indicate the corresponding Smo full length or truncation proteins.

Smo is not phosphorylated by Gprk2 on the known phosphorylation sites

Since Gprk2 is a kinase, it is interesting to test if it phosphorylates Smo directly. There are 26 identified phosphorylation sites on Smo C-tail (Zhang et al., 2004). We made four GST fusion protein GSTSmo555-599, GSTSmo601-700, GSTSmo656-755, and GSTSmo801-899 (the number indicating the start residue and the end residue) to cover those phosphorylation sites (Fig. 5.5 a, b). Then we carried on *in vitro* kinase assay by using recombinant GRK5, which is the closest homolog of Gprk2 in mammals. The dephosphorylated α-Casein was used as a positive control. As shown in Fig. 5.5 c, none of those Smo fragments is phosphorylated by GRK5, suggesting that Gprk2 may regulate

Smo in a manner independent on its kinase activity or through phosphorylating other proteins associated with Smo.

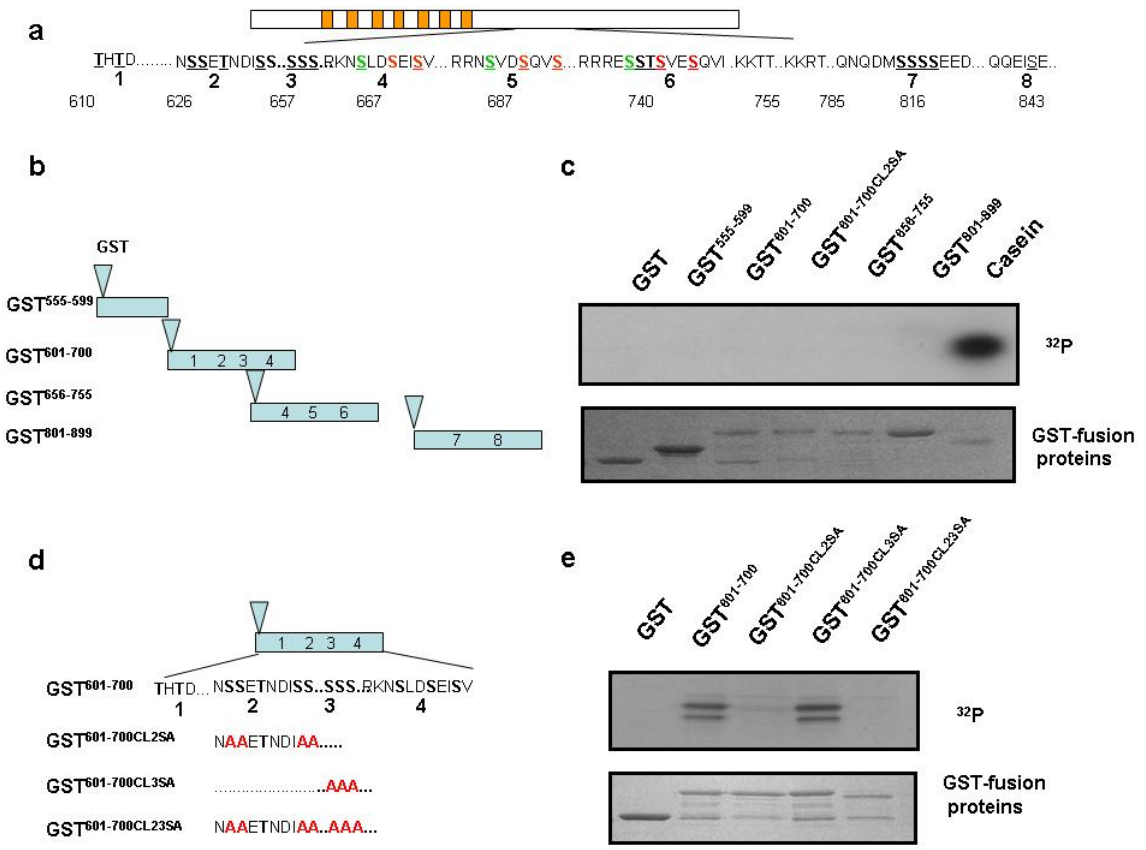



Fig. 5.5- Smo is not phosphorylated by GRK in the *in vitro* kinase assay. a. A diagram shows the phosphorylation clusters (cluster 1 to 8 as indicated) in Smo C-tail. The phosphorylation target sites are indicated with underline. b. A diagram shows various GST fusion proteins. The phosphorylation clusters contained by the fusion protein are labeled by numbers. c. GST fusion proteins immobilized on glutathione beads were incubated with recombinant GRK5 and [γ-$^{32}$P]ATP for 1 hour at 37° C, followed by separating on SDS-PAGE, Comassie Blue staining (lower panel) and autoradiography (upper panel). α-Casien was used as a positive control. None of the Smo fragments is phosphorylated by GRK5. d. A diagram shows GST$^{601-700}$ and the corresponding substitution forms of GST$^{601-700}$. e. GST fusion proteins immobilized on glutathione beads were incubated with recombinant CKI and [γ-$^{32}$P]ATP for 2 hours at 30° C, followed by separating on SDS-PAGE and autoradiography (upper panel). The total GST fusion protein inputs were showed below. CKI phosphorylated GST601-700 at cluster two.

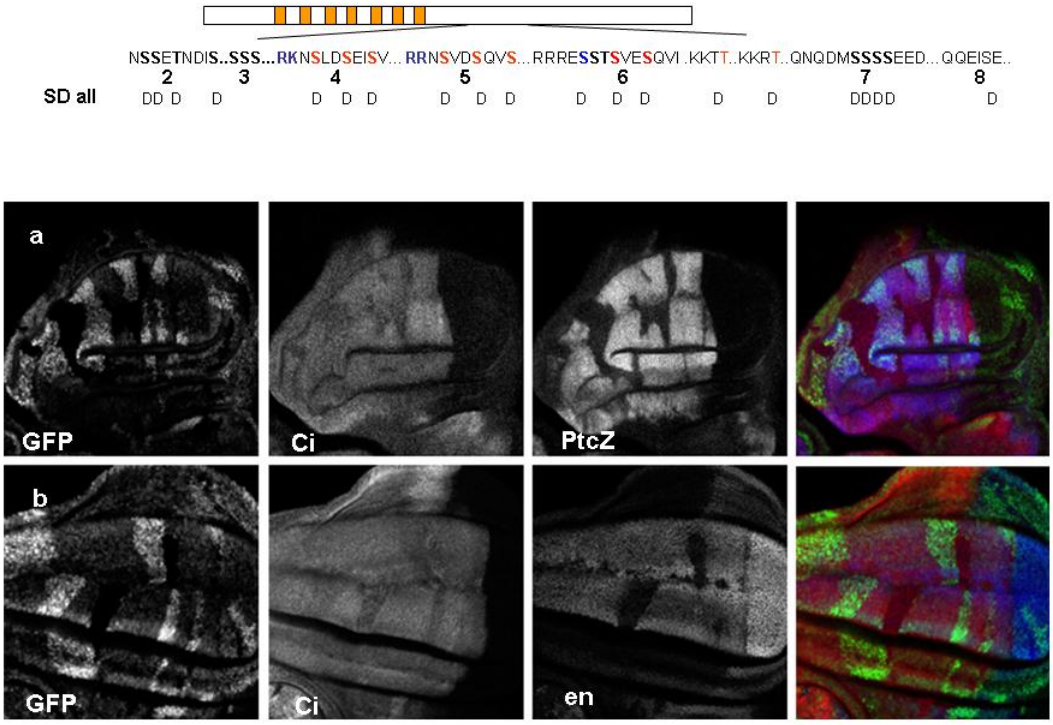



Fig.5.6- The activity of phosphorylation form of Smo still depends on Gprk2. A diagram shows the phosphorylation clusters (cluster 2 to 8 as indicated) in Smo C-tail. SmoSDall with the indicated substitutions is shown underneath. a, b. Wing discs bearing *gprk2* mutant (D15) clones (marked by lack GFP expression) and expressing SmoSDall with *MS1096* were stained to show the expression of GFP, Ci, *ptc-lacZ* (a) or En (b). Inside *gprk2* mutant clones, Ci level and the ectopic expression of *ptc* and En was reduced.

If Gprk2 regulates Smo activity solely by phosphorylating the identified phosphorylation sites, then the phosphorylated form of Smo should immune to loss of Gprk2. However, if Gprk2 regulates Smo activity by other manner independent on Smo phosphorylation, the activity of the phosphorylated form of Smo should still be affected by loss of Gprk2. To test these possibilities, we generated a Smo variant, SmoSDall,

with most of the identified phosphorylation sites mutated to Asp. Overexpression of SmoSDall resulted in constitutive Hh pathway activation, as indicated by high levels of ectopic expression of *ptc* and *en* (Fig. 5.6 a, b outside of the clones). Then, we generate *gprk2* mutant clones in the wing discs with overexpressing SmoSDall. Interestingly, the ectopic expression of *ptc* and *en* driven by SmoSDall diminished in the *gprk2* mutant clones (Fig. 5.6 a, b), indicating that the activity of SmoSDall is still regulated by Gprk2. These data imply that Gprk2 may regulate Smo activity in a manner independent phosphorylation of Smo C-tail by Gprk2.

CKI phosphorylation on a specific cluster in Smo-C tail is necessary for Smo subcellular localization and activity.

In an effort to determine whether other phosphorylation sites in Smo is required for Smo activity in addition to those PKA and CKI sites we identified before (chapter three), we found that the second phosphorylation cluster appears to be important for Smo activity. We generated a CFP tagged Smo mutant with all the Ser in cluster 2 mutated to Ala (SmoCL2SACFP), and overexpressed it in wing discs using *MS1096 Gal4*. As shown in Fig. 5.7 c, SmoCL2SACFP was less active than the wild type SmoCFP (Fig. 5.7 b), as it failed to induce ectopic *dpp* expression. Interestingly, we found that the cell surface level of SmoCl2SACFP(Fig. 5.7 e) was also higher than SmoCFP (Fig. 5.7 d), indicating that the phosphorylation of the cluster two may promote Smo endocytosis.

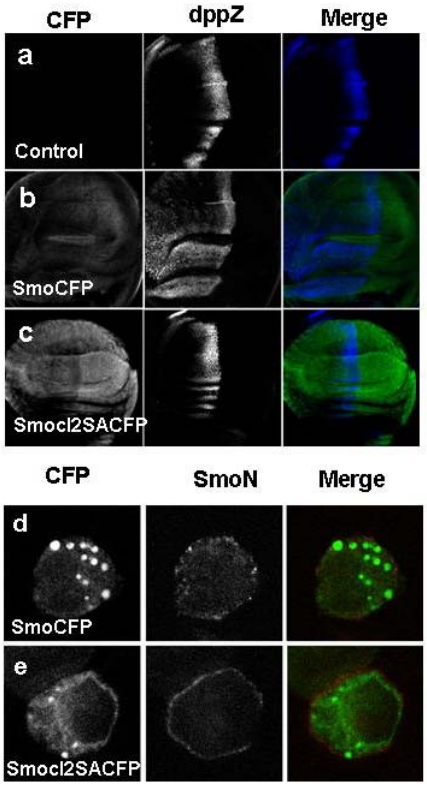



Fig. 5.7-The phosphorylation on cluster two is important for Smo activity and subcellular localization. a-c. Wing discs with or without expressing of SmoCFP or SmoCl2SACFP drove by *MS1096* were stained to show the expression of CFP and *dpp-lacZ*. SmoCFP (b) but not Smocl2SACFP (c) induced ectopic *dpp* expression in the anterior compartment far away A/P boundary. d, e. S2 cells were transfected with SmoCFP or Smocl2SACFP. The cell surface levels of Smo (either wild type or mutant) were detected by Smo cell surface staining. Smocl2SACFP has higher cell surface level than SmoCFP.

We Then investigated which kinase phosphorylates this cluster. As showed in Fig. 5.5 d and e, GSTSmo600-700 was phosphorylated by CKI in the *in vitro* kinase assay. Mutating the phosphorylated Ser in the cluster 2 but not in cluster 3 abolish the phosphorylation by CKI. These data suggest that the cluster 2 probably is phosphorylated by CKI. Consistent with this finding and the high cell surface levels of SmoCl2SA, both Kalderon's (Apionishev et al., 2005) group and our group (unpublished data) found that completely knock down CKIα elevates Smo level in wing discs. These data suggest that CKI phosphorylate Smo at cluster 2, which induces Smo endocytosis and enhance Smo activity.

## Discussion

In summary, we found Gprk2 is essential for Smo activity. In addition, we found CKI phosphorylates the second phosphorylation cluster in Smo CT, which is important for Smo subcellular localization and activity.

How does Gprk2 regulate Hh signaling activity?

In this study, we found that loss of Gprk2 results in reduction of high threshold Hh target gene expression, indicating that Gprk2 is essential for Hh signal transduction. Then the question becomes how Gprk2 regulates Hh signaling and what is the direct target of Gprk2.

Smo shares the same topologic structure with G protein coupled receptor and the vertebrate Smo is regulated by GRK (Chen et al., 2004; Wilbanks et al., 2004), thus Smo is the best potential target of Gprk2. Loss of Gprk2 results in the elevation of Smo level in anterior compartment especially in the region near A/P boundary, suggesting that Smo probably directly regulated by Gprk2. Consistent with this, we found Smo C-tail forms complex with Gprk2. However, no GRK mediated Smo phosphorylation was found. To test whether kinase activity of Gprk2 is dispensable for Smo activity regulation, we generated a kinase dead Gprk2 by mutating two Lys in the ATP binding pocket to Met (Ferguson et al., 1995). To test whether Gprk2KM is indeed a kinase dead form, in vitro kinase assay was performed using immunoprecipitated either FgGprk2 or FgGprk2KM complexes. As we expected, FgGprk2 but not FgGprk2KM phosphorylated α-Casein, indicating that FgGprk2KM is indeed a kinase dead form (Fig. 5.8 a). Then we introduced kinase dead form of Gprk2 (Gprk2KM) in *gprk2* mutant background and test whether it could rescue *gprk2* phenotype. As shown in Fig. 5.8 b-d, the kinase dead form

of Gprk2 rescues *gprk2* mutant phenotype in a same manner as the wild type Gprk2, indicating the kinase activity of Gprk2 is not really critical for regulating Smo activity. However, since both the wild type Gprk2 and Gprk2KM were overexpressed, the slight activity difference between Gprk2 and Gprk2KM might be masked.

Since Gprk2 may regulate Smo independent on phsophorylating Smo, Gprk2 may recruit other molecules such as β-Arrestin to Smo by interacting with both proteins physically. Alternatively, the interaction between Gprk2 and Smo may change Smo conformation and facilitates its interaction with downstream complex in an active manner. However, our data can not exclude the possibility that Gprk2 also directly regulates other components such as Fu, Cos2 *et al.* in Hh pathway. Further study may reveal these aspects.

Endocytosis and cell surface accumulation: two parts of one story

It has been suggested that GRK and β-Arrestin2 mediated vertebrate Smo endocytosis is required for Smo activity (Chen et al., 2004). The elevation of Smo in *gprk2* mutant clones may reflect blocking of endocytosis of Smo. It is possible that endocytosis is also required for Smo activity in *Drosophila*. This may explain why Smo is accumulated but fails to activate Hh pathway in *gprk2* clones.

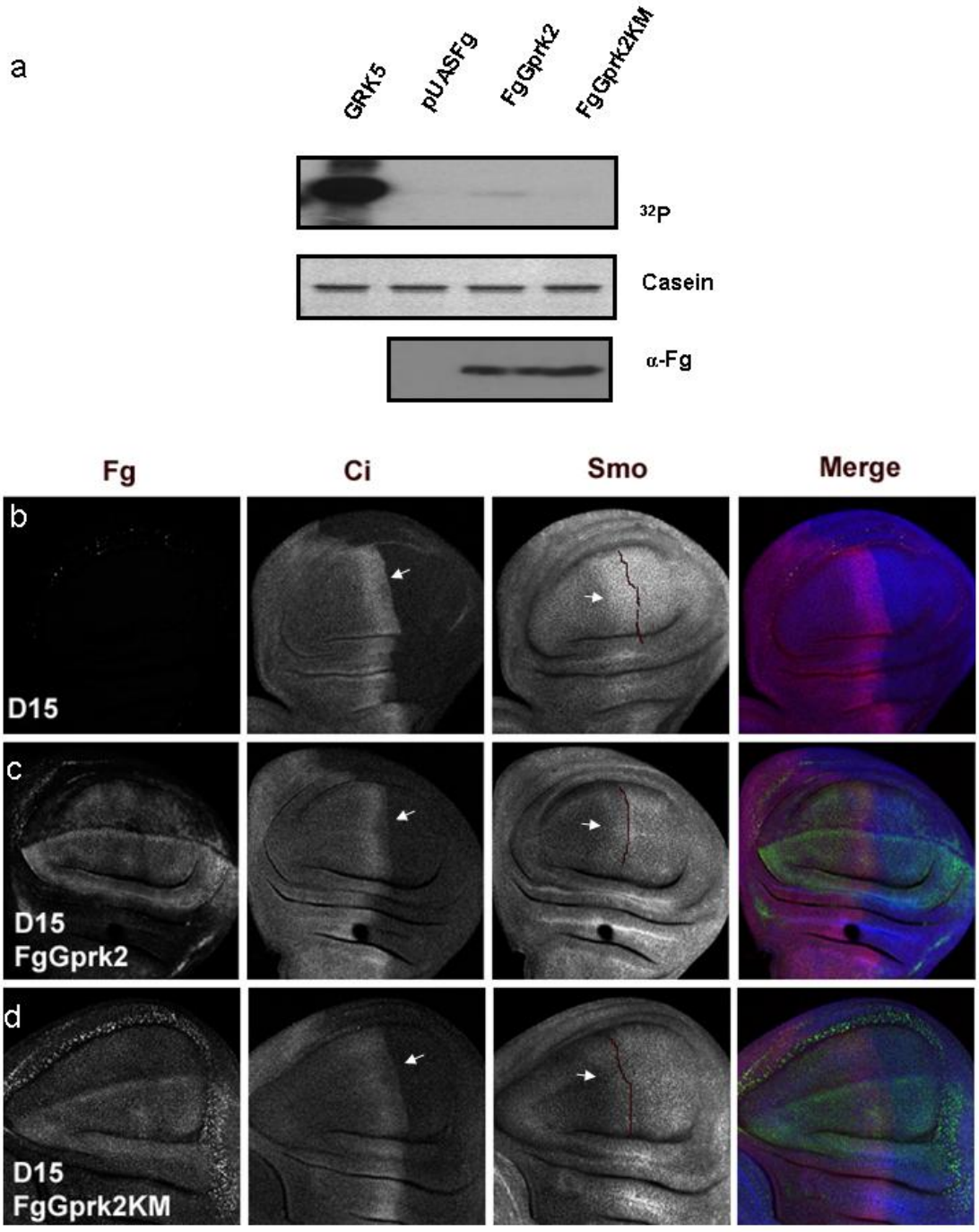



Fig. 5.8- The overexpression either Fg-Gprk2 or Fg-Gprk2KM restored the low Ci region abutting A/P boundary and reduced Smo expansion in A-compartment cells. a. Cell extracts were prepared from S2 cells transfected with either Fg-Gprk2, Fg-Gprk2KM or pUASFg (serve as negative control). Fg-Gprk2 and Fg-Gprk2KM were purified and immobilized on protein A beads from S2 cell lysates by immunoprecipitation with anti-Fg antibody, followed by incubated with α-Casien and [γ-$^{32}$P]ATP for 1 hour at 37° C. The supernatants were separated on SDS-PAGE and stained with Commassie Blue

(middle panel), followed by autoradiography (upper panel). 5% cell lysates were analyzed by western blotting to show the kinase input (lower panle). GRK5 was used as a positive control. FgGprk2 but not FgGprk2KM phosphorylates Casein protein. D15 homozygote Wing discs with or without (b) expressing of Fg-Gprk2 (c) or Fg-Gprk2KM (d) drove by *MS1096* were stained to show the expression of Fg, Ci and Smo. D15 mutants have elevated Ci level abutting the A/P boundary (normally the "low Ci" region, indicated by arrowes) and expension of high level of Smo expression domain (indicated by arrows).

Smo is accumulated on cell surface in response to Hh, while, endocytosis appears to uptake cell surface Smo. It seems that endocytosis and cell surface accumulation of Smo is conflict to each other. Mutating cluster 2 phosphorylation sites to Ala resulted in a increase in Smo cell surface expression and reduction of activity. Mutating PKA or CKI phosphorylation clusters (cluster 4-6) leads to reduction of Smo cell surface accumulation and activity. It is possible that the overall cell surface Smo level is determined by the fates of the endocytosed Smo protein. In the absence of Hh, Smo goes to lysosome and gets degredated after being endocytosed. With the stimulation of Hh, the endocytosed Smo undergoes recycling and reappears on cell surface. It is possible that the endocytosis of Smo consistently exists as observed in wild type Smo uptaking experiment without Hh treatment (chapter three), while the phosphorylation of Smo on the PKA and CKI sites switches the fate of the endocytosed Smo and both processes are required for Smo activity. Based on this model, in the anterior cells, Smo is keeping endocytosed and degradated in lysosomes, whereas in posterior Hh expressing cells, the majority of Smo undergoes recycling in stead of degradation after being endocytosed.

Why endocytosis of Smo is required for Smo activity? As we showed before (Jia et al., 2003), Smo interacts with Cos2-Fu complex and transduces Hh signal. Since the majority of Cos2 appears to associate with endosomes (Stegman et al., 2004), the

endocytosis of Smo may increase the chance of Smo to meet Cos2 and facilitate the reloading of Cos2-Fu complex to Smo.

More work should be done to establish the role of Smo endocytosis in Smo activity regulation. It will be interesting to know whether blocking the general endocytosis machinery will diminish Hh responses or not. However, the complicated issue here is the reception of Hh is affected by loss of endocytosis machinery. So, vigorous experiments should be done to understand the roles of Smo endocytosis in Hh signal transduction.

CKI plays multiple roles in Smo regulation.

In the previous study, we found CKI phosphorylates Smo at three clusters (cluster 4-6) which are pre-phosphorylated by PKA. In addition, we provided evidence that the phosphorylation at CKI sites is essential for Smo activity and cell surface expression. However, CKI knock down in wing discs gave us puzzling results. Slight knock down CKI results in the reduction of Smo level (Jia et al., 2004), while, complete knock down of CKI leads to elevation of Smo level in A-compartment cells. Here, we provided evidence that CKI phosphorylates cluster 2 in Smo C-tail. The S to A mutation in this cluster resulted in a increase in Smo cell surface expression. These data suggest that CKI plays multiple roles in regulating Smo. On one hand, PKA primed CKI phosphorylation is regulated by Hh and is required for Smo cell surface expression and activity. On the other hand, CKI may phosphorylate cluster 2 in Smo C-tail and mediate Smo endocytosis constitutively, suggested by higher cell surface level of Smocl2SA than that of Smo wild type in the absence of Hh. Interestingly, the phosphorylation on cluster two appears to be the premise of Smo activity.

PKA primed CKI phosphorylation sites are classic CKI phosphorylation sites pSXXS (where pS refers to the primed phosphorylation on the Ser residue; X refers to any amino acid; the underline refers to target site). However, the phosphorylation sites in cluster 2 (DLNpSpSEpTNDIpSS: where pS or pT refers to the sites were phosphorylated) are not typical CKI phosphorylation sites. In most cases, CKI sites phosphorylation requires priming by other kinases. However, CKI also can phosphorylate a related unprimed site, which optimally contains a cluster of acidic amino acids at the N-terminus of the target S/T including an acidic residue at $n - 3$ and a hydrophobic region at the C-terminal to the target S/T (Flotow and Roach, 1991; Pulgar et al., 1999). Obviously, our sites are also not optimal unprimed sites although some sites are indeed close to this category. In the case of β-catenine or NF-AT phosphorylation (Amit et al., 2002; Liu et al., 2002; Zhu et al., 1998), CKI phosphorylates first Ser in the sequence of S-L-S, which followed by an acidic clusters. These different phosphorylation patterns of CKI sites indicate that the phosphorylation of CKI is not restricted to a particular sequence. However, our data cannot exclude the possibility that other kinases may also involved in phosphorylate the cluster two *in vivo*.

In this study we provide evidence that cluster 2 phosphorylation is essential for Smo activity, but it is still unclear how cluster 2 phosphorylation regulates Smo activity. Our data suggest that phosphorylation on those sites may promote Smo endocytosis. Interestingly, our lab recent study indicates that the non-visual Arrestin (Krz) may recognize this phosphorylation site and mediate Smo endocytosis (Chen, YB, unpublished data). It will be interesting to know whether loss of function of Krz results in diminishing of the Hh signaling.

## Materials and Methods

Plasmid constructions and transgenes

pUASFgGprk2 was generated by amplifying *gprk2* coding sequence from *Drosophila* embryo cDNA library and subcloning into PUASTFg vector. Site direct PCR mutagenesis was used to construct pUASFgGprk2KM. To construct *UAS-Gprk2RNAi*, a genomic DNA fragment with coding sequence for Gprk2 aa514-714 was cloned into pUAST with the corresponding cDNA fragment inserted in a reverse orientation. pUASSmoSDallFg, pUASSmoCl2SACFP, pUASSmoCl3SACFP and PUAsSmoCl23SACFP were generated by site directed mutagenesis and subcloned into either pUASFg or pUASCFP vector. To generate pGEXSmo555-599, pGEXSmo601-700, pGEXSmo656-755 and pGEXSmo801-899, corresponding Smo cDNA fragments were amplified by PCR and subcloned into pGEX4T-2 vector. To generate pGEXSmo601-700-Cl2SA, -Cl3SA, or-Cl23SA, we used pUASSmoCL2SACFP, -Cl3SACFP or –Cl23SACFP as templates. The corresponding Smo sequences were amplified by PCR and subcloned into pGEX4T-2 vector.

*MS1096*, *ap-Gal4, dpp-lacZ and ptc-lacZ* have been described. Un-precise excision of P-element was performed as described. Clones of mutant cells were generated by *FLP/FRT*-mediated mitotic recombination as described. Genotypes for generating clones are as follows: *ywhsFLP; FRT82 1035/FRT82 GFP* and *MS1096; FgSmoSDall/Cyo[$y^+$]; FRT82GFP/FRT82D15*. Genotypes for rescue experiment are as follows: *MS1096; FgGprk2/Cyo[$y^+$]; FRT82D15/HM* and *MS1096; FgGprk2KM/Cyo[$y^+$];FRT82D15/HM*.

Cell culture, immunoprecipitation, immunoblot, and immunostaining

Cell culture, transfection, immunoprecipitation, western blot and immunostaining were carried out as described in Chapter two, Chapter Three and Chapter Four.

*in vitro* kinase assay

To determine whether FgGprk2KM is indeed a kinase dead form, FgGprk2, FgGprk2KM or control vector was transfected to S2 cells. To prepared cell lysate, cells were incubated with the following buffer: 20 mM Tris/HCl, pH 7.6, 0.5% (v/v) NP40, 250 mM NaCl, and protease inhibitor tablets (Roach), for 15 min on ice. Lysates were then removed and centrifuged at 20,000*g*, at 4°C, for 5 min. The supernatant was removed and added to either mouse anti-Flag antibodies (1:100). Samples were left on ice for 1 h before the addition of protein A beads. After 30 min of rolling at 4°C, samples were washed twice with 10 mM Tris, pH 7.4, 1 mM EDTA buffer and once with kinase buffer (20 mM Tris, pH 7.5, 2 mM EDTA, and 5 mM $MgCl_2$). After the final wash, the beads were resuspended in reaction mix (5 μg of dephosphorylated α-casein, 10 μM unlabeled ATP, 5 μCi of [$\gamma$-$^{32}$P]ATP, and kinase buffer to a total reaction volume of 20 μl) and left to incubate for 1 hour at 37°C. The reaction was terminated by adding in 4× SDS-PAGE loading buffer. Samples were separated by SDS-PAGE electrophoresis. Gels were dried and the extent of phosphorylation was assessed by autoradiography. To determine which part of Smo C-tail was phosphorylated by GRK, the condition was same as mention above, instead of using immunoprecipited kinase, the recombinant GRK5 (upstate) was used. The CKI kinase assay was carried out as described in chapter three.

# CHAPTER SIX

## Conclusions and Future Directions

Seven transmembrane protein Smo is essential for Hh signaling transduction. However, how Smo transduces signal to downstream signaling components is a long standing question in the filed. This study presented evidence that Smo C- tail interacts with downstream Cos2-Fu complex physically, which is essential for Hh signal transduction. However, the interaction itself is not sufficient to activate Hh responses. This study filled the gap between the upstream transducer and the downstream effectors in Hh pathway and provided an explanation as to how Cos2 performs its positive role in Hh signal transduction. However, this study did not answer how the interaction between these proteins transmits Hh signal. We provided evidence that Hh stimulation does not change the binding affinity between Smo and Cos2-Fu complex. But, Hh stimulation does increase Smo level and therefore proportionally increases the total amount of Smo engaged in Smo-Cos2-Fu interaction. In addition, Hh stimulation promotes Smo cell-surface accumulation which changes the distribution of Cos2-Fu complex. It will be interesting to know if Hh stimulation also modifies the interaction mode of Smo-Cos2-Fu complex in future. The FRET assay probably could be used to monitor the differences of interactions with or without Hh treatment. Another interesting experiment could be done is to artificially tether Cos2-Fu complex on the cell membrane and test if that will affect their phosphorylation and activity.

In response to Hh, Smo is phosphorylated and accumulated on the cell surface (Denef et al., 2000). In this study, we provided evidence that PKA and CKI phosphorylate Smo at multiple phosphorylation clusters in response to Hh and the

phosphorylation is essential for Smo cell surface expression and activities. Furthermore, we demonstrated that the levels of Smo phosphorylation correlates with its levels of cell surface expression and activities. We proposed a model that the concentration gradient of Hh regulates Smo activity by progressively phosphorylating Smo. In the future, raising site specific phosphorylation antibodies will help to test this model directly. Another puzzle raised by this study is how Smo phosphorylation is regulated by Hh. Hh stimulation may regulate kinase activity locally, regulate the exposure of the phosphorylation sites, or inhibit the activity of phosphatase. It will be worthwhile to test these possibilities in future.

In this study, we have identified an auto-inhibitory domain (SAID) in Smo C-tail that inhibits Smo cell surface expression and activity. We uncovered multiple Arg clusters in SAID that are essential for the negative regulations. Our data showed that the numbers of Arg clusters is inversely correlated with the cell surface level and activity of Smo. We also provided evidence that phosphorylation counteracts the negative effects by neutralizing the positive charges carried by the Arg clusters. Furthermore, we demonstrate that the Arg clusters inhibit Smo at several levels. First, they keep Smo in a closed inactive conformation mediated by electrostatic interactions. Second, they prevent close proximity of Smo C-tails, which is essential for Smo activity. Third, they prevent Smo cell surface accumulation. All these layer of inhibition could be counteracted by Smo phosphorylation. Increasing of Smo phosphorylation gradually neutralizes the negative effects by multiple Arg clusters, leading to a progressive increase in Smo cell surface expression and activity. However, this study did not provide an explanation as to how Smo cell surface accumulation is regulated by Arg clusters and phosphorylation. In

this study, we showed that Smo aa661-818 is sufficient to prevent FzSmo chimera protein cell surface accumulation, whereas the SD mutation in this fragment restores the cell surface expression of the chimera protein. In future experiments, it will be interesting to identify some interaction partners by using this small fragment of Smo (either wild type or the SD mutant) to perform yeast-two-hybrid or affinity purification.

Finally, we also demonstrated that Gprk2 and CKI regulate Smo activity probably by regulating Smo trafficking. We showed that CKI phosphorylate a specific cluster in Smo C-tail, which is required for Smo subcellular localization and activity. Future study will investigate the detail trafficking regulation of Smo.

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# VITAE

Chao Tong was born in Baotou, Inner-Mongolia, China, on September 18th, 1977. In 1995, she entered NanKai University in Tianjin, China. She received the degree of Bachelor of Science with a major in Biochemistry and Molecular Biology from Nan Kai University in June 1999. At the summer of 1999, she entered the graduate program at Chinese Academics of Science. In 2000, she joined Dr. Qingyuan Sun's lab in Institute of Zoology, Chinese Academics of Science as a graduate student. In June 2002, she received the degree of Master of Science with a major in Physiology. In August, 2002, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. In 2003, she joined the Genetics and Development program and began her doctoral research in the lab of Dr. Jin Jiang.

Permanent Address: ZiYou Lu 13 Jiefang 6 Dong #7,
QingShanQu, BaoTou, Inner-Mongolia, China, 014030