DISSECTION OF MECHANISMS REGULATING

THE *DROSOPHILA* HEDGEHOG PATHWAY

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TO MY BELOVED

GRANDPARENTS & PARENTS

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DISSECTION OF MECHANISMS REGULATING THE *DROSOPHILA* HEDGEHOG PATHWAY

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DISSECTION OF MECHANISMS REGULATING

***DROSOPHILA* HEDGEHOG PATHWAY**

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Hedgehog (Hh) signaling is essential for both embryonic development and adult tissue homeostasis. Malfunction of Hh signaling pathway causes many human disorders including birth defects and cancers. In *Drosophila*, the G-protein-coupled-receptor-like protein Smoothened (Smo) transduces the Hh signal across the plasma membrane, and an intracellular Hh signaling complex (HSC) containing the kinesin-related protein Costal2 (Cos2), the serine/threonine protein kinase Fused (Fu) and a PEST-domain containing protein suppressor of Fused (Sufu) relays the Hh signal downstream from Smo to the Zn finger transcription factor *Cubitus interruptus* (Ci).

Our previous studies have demonstrated that Hh transduces signal by regulating the subcellular localization and conformational state of Smo, but how Smo relays the signal to cytoplasmic signaling

components remains poorly understood. In this study, we show that Hh-induced Smo conformational change promotes the recruitment of Cos2/Fu complex and Fu dimerization. We find that induced dimerization through the Fu kinase domain activates Fu by inducing multi-site phosphorylation of its activation loop (AL), and phospho-mimetic mutations of AL suffice to activate the Hh pathway. Moreover, we find that activated Fu regulates Ci by both promoting its transcriptional activator activity and inhibiting its proteolysis into a repressor form. We provide evidence to suggest that activated Fu exerts the regulation by interfering with the formation of Ci-Sufu and Ci-Cos2-kinase complexes that normally inhibit Ci activity and promote its processing.

In the rest part of the study, we further explore additional mechanisms regulating Ci activity. We have identified and characterized three types of functional regulatory elements in Ci, including a transcriptional repression domain in the N-terminal region of Ci, multiple Ser/Thr motifs in the amino-(N-) and carboxy-(C-) terminal regions of Ci serving as HIB/SPOP E3 ligase-specific degrons, and finally a novel PY-NLS around the N-terminal highly conserved domain of Ci.

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A-	Anterior-
A/P	Anterior/Posterior
aa	amino acids
AG4	*actin-Gal4*
AL	Activation loop
AP	After photobleaching YFP
BP	Before photobleaching YFP
C-	Carboxy-
CBP	CREB-Binding Protein
CC	Coiled-coil dimerization motif
CCm	Dimerization-deficient CC mutant motif
Ci	*Cubitus interruptus*
Ci^A	Ci hyperactive form
Ci^F	Ci full-length form
CiGA	Ci-Gal4 fusion protein
CiN	Ci N-terminus, aa 1-440
Ci^R	Ci repressor form
Ci^U	Ci processing-deficient form
Cos2	Costal2
CRD	Cysteine-rich domain
C-tail	Carboxy-terminal tail
Dhh	Desert hedgehog
Disp/Disp1	Dispatched
dsRNA	Double-stranded RNA

ECM	Extracellular matrix
EXT	Exostosin
FBS	Fetal bovine serum
Fg	Flag
FKBP	FK506 binding protein
FRET	Fluorescence resonance energy transfer
Fu	Fused
fu0	Class 0 *fu* allele
fuI	Class I *fu* allele
fuII	Class II *fu* allele
Fz	Frizzled
GA	Gal4 activation domain
GAG	Glycosaminoglycan
GPCRs	G protein coupled receptors
HAT	Histone acetyltransferase
HDAC	Histonedeacetylase
Hh	Hedgehog
Hh-N	Hh N-terminal fragment
HIB	Hh-induced Math and BTB domain containing protein
HS	Heparin sulfate proteoglycan
HSC	Hh signaling complex
HSPGs	Heparin sulfate proteoglycans
IFT	Intraflagellar transport
Ihh	Indian hedgehog
IP	Immunoprecipitation
Kapβ	Importin/karyopherin β

LMB	Leptomycin B
N-	Amino-
NES	Nuclear export signal
NLS	Nuclear localizaion signal
NLS^{C}	classic bipartite NLS of Ci
NLS^{PY}	PY-NLS of Ci
NR	N-terminal regulatory element
NRD	N-terminal repression domain
P-	Posterior-
PI4P	Phospholipid phosphoatidylinostitol-4 phosphate
PP1	Protein phosphotase 1
PP2A	Protein phosphotase 2A
PP4	Protein phosphotase 4
Ptc	Pathed
Ptc-Hh	Hh-bound inactive form of Ptc
ptc-luc	ptc-luciferase
$Ptc^{\Delta loop2}$	Hh-binding deficient active form of Ptc
RND	Resistance-nodulation cell division
SAID	Smo auto-inhibitory domain
Shh	Sonic hedgehog
Smo	Smoothened
SSD	Sterol-sensing domain
Sufu	Suppressor of Fused
Trn	Transportin
WB	Western Blot
ZPA	Zone of polarizing activity

λ -pp	λ-protein phosphotase

CHAPTER I

Introduction

1. Hedgehog (Hh) signaling pathway overview

The Hh signaling pathway is one of the evolutionarily conserved signaling cascades essential for both embryonic development and adult tissue homeostasis (Jiang and Hui 2008, Varjosalo and Taipale 2008). In insects, Hh is a central patterning signal controlling the development of wing (Mohler 1988), leg (Diaz-Benjumea et al. 1994), and eye discs (Heberlein et al. 1995), as well as regulating germ-cell migration (Deshpande et al. 2001). In vertebrate embryos, Hh is expressed in three key signaling centers, including the notochord, the floor plate, and the zone of polarizing activity (ZPA), thus playing key roles in neural tube and limb development (Placzek 1995, Tabin 1991). In addition, Hh signal also directs the formation and the persistence of certain stem- and precursor-cell populations to maintain adult tissue homeostasis (Lai et al. 2003, Reya et al. 2001, Zhang Y. and Kalderon 2001). Not surprisingly given the important roles of Hh pathway in many biological processes, misregulation of Hh signal has been associated with various human diseases (Taipale and Beachy 2001). Reducing Hh signal in human embryos causes birth defects, such as holoprosencephaly (Chiang et al. 1996, McMahon et al. 2003); whereas aberrant activation of Hh pathway in adults leads to multiple types of human cancers (Pasca di Magliano and Hebrok 2003).

2. The Hh ligand

The single *Drosophila hh* gene was first identified by screening for genes essential for embryonic segment polarity (Nusslein-Volhard and Wieschaus 1980). Later, three *hh* homologies, *Sonic hedgehog (Shh)*, *Indian hedgehog (Ihh)*, and *Desert hedgehog (Dhh)*, were identified in mammals (Bitgood et al. 1996, Chiang et al. 1996, St-Jacques et al. 1999).

The Hh family genes encode unusual secreted proteins (Lee J. J. et al. 1992), which serve as ligands at the upstream of the signaling cascade. It is well known that Hh can acts as a classical morphogen that controls cell fates within a target field as a function of its concentration. For example, in *Drosophila* wing imaginal discs, Hh acts as a short-range morphogen that controls three alternative cell fates in a field spanning over 10~15 cell diameters (~20μm) (Strigini and Cohen 1997); in vertebrate neural tubes, Hh acts as a long-range morphogen that controls several cell fates in a field spanning over ~200μm (Jacob and Briscoe 2003). Nevertheless, there are also cases that Hh can function as a mitogen regulating cell proliferation or as an inducing factor controlling the form of a developing organ. For example, Ihh regulates proliferation and differentiation of chondrocytes and is essential for bone formation (St-Jacques et al. 1999).

Although Hh has diverse effects in different contexts, the basic process of Hh production, secretion and transportation is highly conserved among different species. In general, Hh proteins are first synthesized as ~45-KD precursor proteins and targeted to the endoplasmic reticulum and Golgi (Lee J. J. et al. 1994). Then Hh undergoes an intramolecular cleavage to yield a 25-KD C-terminal fragment and a ~19-KD N-terminal fragment (referred to as Hh-N) (Bumcrot et al. 1995). The resulting Hh-N fragment is further modified by covalently adding a cholesterol moiety to its C-terminus (Porter et al. 1996a, Porter et al. 1996b) and a palmitic acid to its most N-terminal cysteine residue to become a mature signaling molecule (Chen M. H. et al. 2004a). This dually lipid-modification is essential for Hh-N activity: cholesterol is important in Hh oligomerization and its packaging into signaling complexes, whereas palmitate is required for high-level Hh signaling activity (Chen M. H. et al. 2004a, Panakova et al. 2005, Zeng et al. 2001).

After modification, Hh is packaged into lipid-associated particles, releases from Hh-producing cells, and travels many cell diameters to form a concentration gradient. Genetic analysis in *Drosophila* has revealed that a twelve-transmembrane domain Patched (Ptc) -related protein Dispatched (Disp/Disp1)

participates in Hh release from Hh-producing cells (Burke et al. 1999). In *disp* mutants, although the production and processing of Hh are normal, Hh-producing cells fail to secrete Hh. As a consequence, Hh accumulates at high levels in Hh-producing cells, only signals to the cells adjacent to the Hh-producing cells and no long-range signaling event is observed (Burke et al. 1999). Disp contains a sterol-sensing domain (SSD), which is often involved in the regulation of protein subcellular localization and vesicle trafficking in cholesterol homeostasis and cholesterol linked signaling (Kuwabara and Labouesse 2002), implying that Disp may function through regulating vesicle trafficking.

The mechanism responsible for Hh spreading is still mysterious. Former observations that Hh is mainly located extracellularly in the receiving tissues and that the transport of Hh is facilitated by inhibiting endocytosis (Gallet and Therond 2005, Han C. et al. 2004a, Torroja et al. 2004) argue against the model that Hh is transported by transcytosis, a common strategy adopted by many other morphogens (Tabata and Takei 2004). Instead, current evidence strongly supports that Hh is diffusing through the extracellular matrix (ECM), which is facilitated by several heparin sulfate proteoglycans (HSPGs), extracellular molecules with a protein core to which heparin sulfate (HS) glycosaminoglycan (GAG) chains are attached (Han C. et al. 2004b, Kramer et al. 2003). Consistent with this, several genes belonging to Exostosin (EXT) family which is involved in HSPGs biosynthesis, modification and processing have been shown to regulate Hh signaling. For example, *ttv, sotv, and botv* play important roles in HS-GAG polymerization and HS chain initiation and elongation (Bellaiche et al. 1998, Bornemann et al. 2004, Takei et al. 2004); while *sulfateless, sugarless, fringe connection, slalom, and notum* are important for HSPCs modification and processing (Esko and Selleck 2002, Giraldez et al. 2002, Goto et al. 2001). Interestingly, in the mutant clones of those genes, Hh proteins become unstable (Bornemann et al. 2004), suggesting that HSPGs may have an extra role in stabilizing Hh besides propagating it. In addition to EXT family, the genes *dally* and *dlp* encoding glypicans (Lin 2004), *troll* encoding a perlecan core protein (Lin 2004), and *shf* encoding a WIF domain protein are also reported required for the transport of lipid-modified Hh (Glise et al. 2005, Gorfinkiel et al. 2005, Hsieh et al. 1999).

3. Receiving the Hh signal at cell membrane

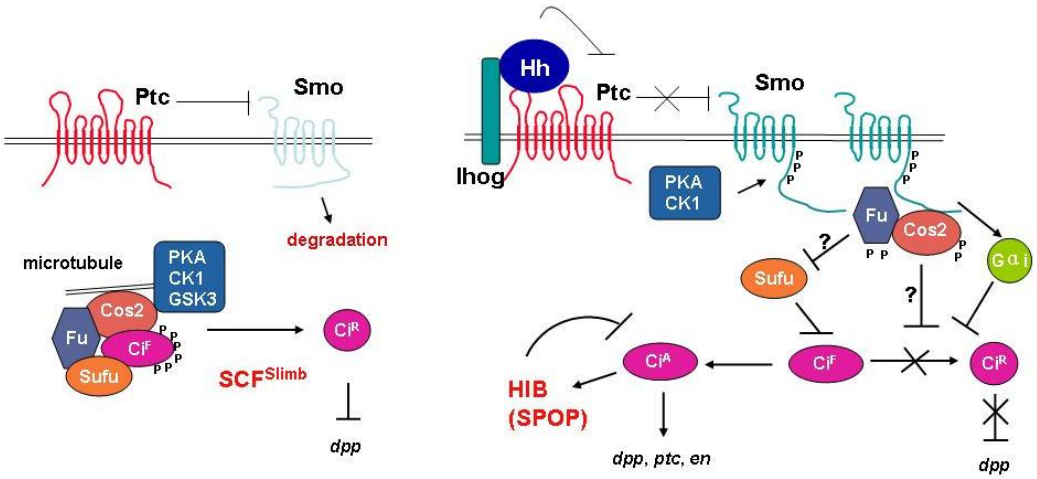


Figure 1.1 The *Drosophila* Hh signaling pathway (Adapted from (Zhang W. et al. 2005)). Left: In the absence of Hh, Ptc inhibits Smo. Cos2 recruits multiple kinases to promote the hyperphosphorylation of full-length Ci (Ci^F), which then targets it for SCF^{Slimb}/proteasome-mediated processing to generate a truncated repressor form (Ci^R) that blocks the expression of *dpp*. Right: Hh binds Ptc and alleviates its inhibition on Smo. Smo accumulates on the cell surface and undergoes a phosphorylation-dependent conformational switch. The interaction of Smo with Cos2 complexes at the cell surface dissociates Cos2-Ci-kinase complexes. Low levels of Hh suffice to block Ci processing either through Gαi or possibly through Fu kinase activity, leading to the derepression of *dpp*. High levels of Hh convert Ci^F to hyperactive Ci^A likely through releasing the Sufu-mediated repression by Fu kinase activity, leading to the activation of Hh-responsive genes, *ptc* and *en*. Ci^A becomes labile due to HIB E3 ligase-mediated degradation, which provides a negative feedback to fine tune the pathway activity.

The *Drosophila* Hh reception system consists of a twelve-transmembrane protein Ptc that acts as the Hh ligand receptor (Chen Y. and Struhl 1996, Stone et al. 1996), a seven-transmembrane protein Smoothed (Smo) that acts as the Hh signal transducer (Alcedo et al. 1996, van den Heuvel and Ingham 1996), and recently identified Ihog/Cdo proteins that function as co-receptors for Ptc (Tenzen et al. 2006,

Yao et al. 2006). In the absence of Hh ligand, Ptc represses the activity of Smo; whereas Hh binding to Ptc/Ihog/Cdo releases its repression on Smo and allows Smo to be activated (Taipale et al. 2002). Due to the difficulties in studying large membrane-associated proteins, several questions on the regulation of Ptc and Smo remain to be answered.

First, although it has been shown that Ptc inhibits Smo by both promoting its turnover and preventing its accumulation at the cell surface (Taipale et al. 2002), how Ptc achieves this inhibition is still elusive. Early reports claimed that Ptc inhibited Smo by directly binding to it; but a later study argued that the observed interaction could be due to overexpression artifacts, and that Ptc in fact acts sub-stoichiometrically to inhibit Smo activity (Stone et al. 1996, Taipale et al. 2002). Consistent with this, overexpression of Smo is not sufficient to activate the pathway (Alcedo et al. 2000, Ingham et al. 2000). Interestingly, a recent study shows that Smo activation is dependent on the levels of the phospholipid phosphoatidylinostitol-4 phosphate (PI4P), and that loss of Ptc causes an increase in PI4P levels, which raises a possibility that Ptc may suppress Smo through directly or indirectly inhibiting the accumulation of PI4P (Yavari et al. 2010). In addition, sequence analysis indicates that Ptc contains a SSD, implying that Ptc might regulate lipid trafficking to repress Smo. In line with this, a recent study shows that *Drosophila* Ptc recruits internalized lipoproteins to Ptc-positive endosomes and regulates Smo degradation by changing the lipid composition of endosomes through which Smo passes (Khaliullina et al. 2009). Moreover, sequence analysis also places Ptc in the resistance-nodulation cell division (RND) superfamily of permeases and transporters, which can form oligomers and usually function as pumps to remove antibiotics, toxic organic compounds, and metal iron from bacteria (McKeegan et al. 2003, Taipale et al. 2002). Importantly, mutating the conserved residues which are required for the function of the bacteria transporter impairs the function of Ptc, which raises another interesting possibility that Ptc may transport some hydrophobic molecules and repress Smo activity likely through either increasing local concentrations of an inhibitor or decreasing levels of an activator (Eaton 2008, Taipale et al. 2002). Consistent with this, several exogenous small molecules have been identified that act as either agonists or

antagonists of vertebrate Smo (Chen J. K. et al. 2002). Currently, some researchers are striving to identify small molecules that function in the physiological condition, while others are using traditional genetic methods to identify the synthesis or modification enzymes for the small molecules to further examine this model.

Second, it is still unclear how Hh attenuates Ptc's repression activity. As a member of RND superfamily proteins, both truncated and full-length forms of *Drosophila* Ptc can trimerize (Lu et al. 2006); thus, it is thought that the binding of Hh to Ptc may disperse and inactivate the Ptc oligomer and, in turn, disrupt the transport of the small molecules (Wilson and Chuang 2010). In addition, Ptc and Hh are found to colocalize to intracellular vesicles in Hh-responding cells both in the *Drosophila* embryo and imaginal disc, suggesting that the internalization of Ptc-Hh complex could be another mechanism to restrict Ptc's activity (Bellaiche et al. 1998, Burke et al. 1999, Martin et al. 2001, Strutt et al. 2001). Particularly, in some cell types, once internalized, both proteins are targeted to the lysosome for degradation (Mastronardi et al. 2000), providing a self-limiting mechanism by which Hh restricts its own range of action. Furthermore, given Ptc regulating Smo trafficking and degradation using lipoprotein-derived lipids, the presence of Hh on lipoproteins may attenuate Ptc's repression by inhibiting the utilization of their lipids by Ptc (Khaliullina et al. 2009).

The third open question is how Smo activity is boosted upon Hh releasing Ptc's inhibition. Smo is most closely related to the Frizzled (Fz) family of G protein coupled receptors (GPCRs) (Alcedo et al. 1996, van den Heuvel and Ingham 1996). Although both Smo and Fz have an N-terminal extracellular cysteine-rich domain (CRD), which, in Fz, is required for binding to Wnt family ligands, Smo appears not to interact directly with its activating ligand, Hh (Nusse 2003). Instead, as mentioned above, Hh abrogates an inhibitory effect exerted on Smo by direct binding to Ptc. Nevertheless, the CRD of Smo is indispensable for Smo's activity in *Drosophila*, because deletion of CRD from *Drosophila* Smo abolished its activity (Alcedo et al. 1996, Nakano et al. 2004). It is thus possible that CRD binds some small

molecules in response to Hh as suggested by the pump model of Ptc. Alternatively, CRD may be critical for the structure integrity or oligomerization of Smo (Zhao et al. 2007a). The cytoplasmic C-terminal tail (C-tail) is also essential for Smo activity. Overexpression of a membrane-tethered version of the Smo C-tail alone can partially activate Hh signaling (Jia et al. 2003). Interestingly, expression of a Fz/Smo chimeric protein, in which the cytoplasmic domain of Fz was replaced with that of Smo, could mediate both low and high levels of Hh responses in response to Wnt family ligands, suggesting that the C-tail is sufficient to mediate all responses to Hh when properly regulated (Hooper 2003). Recent studies have demonstrated that, in response to Hh, Smo C-tail is phosphorylated by multiple kinases including PKA, CK1, Gprk2, and CK2 (Apionishev et al. 2005, Chen Y. et al. 2010, Cheng S. et al. 2010, Denef et al. 2000, Jia et al. 2010, Jia et al. 2004, Zhang C. et al. 2004). These Hh-induced phosphorylation events activate Smo by promoting Smo cell surface accumulation as well as converting Smo from a closed inactive conformation to an open active conformation via disrupting the Arg-motif mediated intracellular interaction in its C-tail (Jia et al. 2004, Zhao et al. 2007a). In addition to phosphorylation, some preliminary evidence suggests that Hh may regulate Smo activity through other types of modification, such as ubiquitination and sumoylation, even though further efforts need to be made to identify the responsible E3 enzyme(s) and to characterize the modification sites in Smo (unpublished data).

Finally, how different concentrations of Hh are perceived and interpreted by the receptor system at the cell membrane is still a mystery. At the level of Ptc, unlike the traditional model of morphogen interpreting in which the absolute number of active receptors reflects information of different morphogen concentrations, previous study suggested that both active and inactive Ptc receptors are important for cells to interpret Hh ligand concerntrations (Casali and Struhl 2004, Lu et al. 2006). By expressing constitutive Hh-bound inactive form of Ptc (Ptc-Hh) and Hh-binding deficient active form of Ptc ($Ptc^{\Delta loop2}$) at different ratios, researchers found that Hh pathway is initiated when the ratio for Ptc-Hh to $Ptc^{\Delta loop2}$ is 3:1 or greater, whereas pathway activity is blocked when the ratio drops to 3:2 or lower. Since Ptc, as a

member of RND family proteins, can trimerize, thus it is likely that multiple Ptc molecules may form a functional complex, and information of Hh concentration can be translated into the ratio of liganded inactive to unliganded active Ptc in one complex, which further controls downstream pathway activity (Casali and Struhl 2004, Lu et al. 2006). As for Smo, our previous study has identified an auto-inhibitory domain (SAID) in *Drosophila* Smo C-tail (Zhao et al. 2007a). A striking feature of the SAID is that it contains multiple regulatory modules each of which consists of an Arg cluster linked to a PKA/CK1 phosphorylation cluster (Zhao et al. 2007a). In the absence of Hh, the positively charged Arg clusters in SAID intramolecularly interact with a negatively charged acidic residue rich fragment in the distal region of Smo C-tail, which results in a folding back of the Smo C-tail to form a closed inactive conformation (Zhao et al. 2007b). Hh-induced phophorylation of PKA/CK1 clusters in SAID can neutralize the adjacent Arg elements, leading to disruption of the intracellular electrostatic interaction and, in turn, switching Smo to an open active conformation with increased *trans*-proximity of Smo C-tails (Zhao et al. 2007a). Interestingly, a progressive change of Smo cell surface accumulation, C-tail conformation and activity was observed when gradually increasing the amounts of phospho-mimetic mutations of PKA/CK1 sites, suggesting that phosphorylation at a given PKA/CK1 cluster may only neutralize the adjacent Arg cluster (Zhao et al. 2007a). Moreover, a recent study showed that protein phosphatase 1 (PP1) directly dephosphorylated PKA-phosphorylated Smo to reduce signaling mediated by intermediate concentrations of Hh, whereas protein phosphatase 2A (PP2A) specifically dephosphorylated PKA-primed, CK1-phosphorylated Smo to restrict signaling mediated by high levels of Hh (Su et al. 2011). Hence, by employing multiple Arg clusters as inhibitory elements and modulating differential phosphorylation status of their adjacent PKA/CK1 clusters, Smo can act as a rheostat to translate graded Hh signals into distinct responses (Zhao et al. 2007a).

4. Cytoplamic Hh signal transducers

Smo has homology to the Fz family of GPCRs, but the role that G proteins may have in the Hh signal transduction is quite controversial. An early large-scale RNAi screen in *Drosophila* cl-8 cells

reported that knocking down Gα or Gγ subunits had no effects on the ability of Hh to activate the downstream transcription factor *Cubitus interruptus* (Ci) (Lum et al. 2003a). However, most recent study in *Drosophila* cl-8 cells revealed that *Drosophila* Gαi is required for full activation of Hh signaling. Consistently, overexpressing a constitutively activated form of Gαi results in ectopic pathway activation in *Drosophila* wing discs. Further study indicated that Hh can mediate the recruitment of Gαi to Smo, and that activation of Gαi results in decreased intracellular cAMP level, implying that Hh may regulate PKA through Gαi-mediated modulation of the intracellular cAMP concentration. Taken together, these results suggest that Gαi can function immediately downstream of Smo to transduce the Hh signal in cytosol (Ayers and Therond 2010, Ogden et al. 2008).

Although recent findings support that Smo can act as a canonical GPCR, for a long time, a widespread speculation is that Smo signals through some novel G-protein-independent mechanisms. Indeed, the identification of a cytosolic Hedgehog signaling complex (HSC), containing Costal2 (Cos2), Fused (Fu) and Suppressor of Fused (Sufu), provides another physical link between Smo and Ci (Apionishev et al. 2005).

cos2 encodes a 1201 amino acid polypeptide that is structurally related to kinesin heavy chain. Its N-terminal region (aa 1-450) is predicted to form a globularly structured microtubule binding domain, with aa 136-389 sharing 25%~30% identity with the motor domains of kinesin family proteins; its central region (aa 643-990), containing 36 heptad repeats, is predicted to mediate the formation of a stable homodimer through a parallel coiled coil motif; and its C-terminal region (aa 1050-1201), also predicted to form globular structure, is thought to function as a cargo domain (Lum et al. 2003b, Robbins et al. 1997, Sisson J. C. et al. 1997). Although Cos2 associates with membrane vesicles (Stegman et al. 2004) and microtubules (Robbins et al. 1997) in a manner regulated by Hh, the function of these interactions is still largely unknown. A recent study showed that Cos2 motility which depends on an active motor domain, ATP and microtubules is required for the Hh pathway activity, and that Cos2 can recruit and

transport other Hh pathway components, indicating that Cos2 can function as a kinesin-like protein in transducing Hh signal (Farzan et al. 2008). In addition, the facts that Cos2 physically associates with Smo (Ogden et al. 2003), Ci (Wang G. and Jiang 2004) and a number of kinases (Monnier et al. 2002, Zhang W. et al. 2005), suggest that Cos2 can also function as a scaffold to assemble the Hh signaling complex. Interestingly, genetic studies revealed that Cos2 plays dual roles in transducing Hh signal. On the one hand, *cos2* mutation results in up-regulation of Hh target gene, *dpp*, expression (Capdevila and Guerrero 1994) and duplication of the appendages indicative of Hh pathway activation (Grau and Simpson 1987), suggesting a negative role of Cos2 in regulating Hh signaling. On the other hand, in *cos2* mutant clones abutting the Anterior/Posterior (A/P) boundary, the expression of Hh target gene, *en,* is compromised (Wang G. et al. 2000b), implying a positive role of Cos2 that is essential for transducing high levels of Hh pathway activity. Further studies suggest that the negative role of Cos2 can be attributed to its abilities 1) to recruit multiple kinases, including PKA, GSK3 and CK1, to phosphorylate Ci, which targets Ci to SCF^{Slimb} mediated proteolytic processing to generate a truncated repressor form (Jia et al. 2002, Jia et al. 2005, Price and Kalderon 2002); 2) to directly interact with both N- and C- termini of Ci, which traps Ci in the cytoplasm and prevents Ci nuclear translocation (Wang Q. T. and Holmgren 2000); and 3) to interact with Smo and block Hh-induced Smo phosphorylation, which provides a negative feedback mechanism to fine tune the pathway activity (Liu Y. et al. 2007). However, how Cos2 functions as a positive regulator remains elusive.

Another critical component of HSC is Fu, a Serine/Threonine protein kinase (Preat et al. 1993). *Drosophila* Fu consists of an N-terminal serine/threonine kinase domain (aa 1-305) and a C-terminal regulatory domain (aa 306-805) (Therond P. et al. 1996a). Using the yeast two-hybrid method and *in vitro* binding assay, a Sufu-interaction domain (aa 306-436) and a Cos2-interaction domain (aa 523-805) were mapped to the C-terminal region of Fu (Fukumoto et al. 2001, Monnier et al. 1998). Consistent with *fu* being a positive regulator essential for converting the latent Hh transcription factor into a hyperactive transcriptional activator, *fu* mutants are embryonic lethal, displaying a segment polarity defect similar to

hh mutants in *Drosophila* (Forbes et al. 1993, Nusslein-Volhard and Wieschaus 1980, Ohlmeyer and Kalderon 1998); and in adult wings of *fu* mutants, the longitudinal veins 3 and 4 are fused, and the double row of marginal bristles posteriorly extend, indicating loss of the highest level of Hh target gene, *en*, expression (Fausto-Sterling 1978, Hidalgo 1994, Sanchez-Herrero et al. 1996, Tabata et al. 1995). Based on the genetic interactions with Sufu (refer to next paragraph), *fu* mutant alleles have been further defined as three classes (Preat et al. 1993, Therond P. et al. 1996a). Class 0 (*fu0*) and Class I (*fuI*) *fu* alleles are completely suppressed for their *fu* mutant phenotypes and behave like wild type in *fu; Sufu* double mutants; whereas Class II (*fuII*) alleles, although suppressed for *fu* mutant phenotypes, display *cos2* mutant phenotypes in *fu; Sufu* double mutants. In addition, Class I mutants are dominant over Class II in *fuI/fuII; Sufu* flies while Class 0 mutants are recessive over Class II in *fu0/fuII; Sufu* flies (Therond P. et al. 1996a). Further molecular characterization reveals that Class 0 alleles have large deletions encompassing the whole *fu* gene and some neighboring genes; Class I alleles have mutations that appear to affect the Fu kinase domain; and Class II alleles encode frame shift mutations that truncate the C-terminal domain of Fu, which abolishes the ability of Fu to interact with Cos2 (Therond P. et al. 1996a). Thus, it seems that both the kinase activity and the association with Cos2 are required for normal function of Fu. Interestingly, besides binding to Cos2 and Sufu, the C-terminus of Fu also interacts with its N-terminal kinase domain (Ascano and Robbins 2004). So far the role of this intra-molecular interaction in regulating Fu activity is still unclear. David Robbins' group found that overexpressing Fu C-tail in wild-type flies causes *fu* mutant phenotypes; and they proposed that Fu C-tail functions in a dominant negative manner, by competing endogenous Fu from binding to Cos2 (Ascano et al. 2002). Nevertheless, a later study from the same group revealed that Fu C-tail appears capable of promoting or disrupting Hh signaling under different genetic contexts, which is determined by a balance between providing enough Fu C-tail to allow the Fu kinase domain to reentry into the HSC and providing too much Fu C-tail that would bind Cos2 or the Fu kinase domain separately. Based on this, they proposed that the Fu C-tail may play dual roles in regulating the Fu activity: on the one hand, the Fu C-tail may act as an auto-inhibitory element and lock the Fu kinase domain in an inactive state via associating with it in the absence of Hh; on the other hand,

the ability of the Fu C-tail to bring the Fu kinase domain to HSC through interacting with Cos2 seems essential for the Fu activation in response to Hh (Ascano and Robbins 2004). Thus, an attractive model for Hh-induced activation of Fu would be promoting a conformational switch of Fu, which disrupts the intra-molecular association and in turn releases the inhibition of Fu C-tail on its kinase domain.

A screening for dominant suppressors of *fu* mutant phenotype identified *Sufu* as another component of HSC (Preat 1992). *Sufu* gene encodes a PEST domain containing protein, which does not show any significant homologies to other known proteins (Pham et al. 1995). Although the PEST domain is usually involved in protein degradation (Rechsteiner and Rogers 1996), and there are observations that mammalian Sufu undergoes rapid degradation in certain cancer cells and that Shh signaling can promote ubiquitination and destruction of Sufu in proteasomes in certain cell lines (Chen Y. et al. 2011a, Yue et al. 2009), whether controlling the turnover of Sufu protein is important for regulating the Sufu activity *in vivo* is still uncertain. Given the observation that *Sufu* mutant is capable to reverse *fu* mutant phenotypes, it is thought that *Sufu* acts as a negative effecter preventing the Ci activation, and Fu kinase activity is required to release this inhibition in response to Hh (Ohlmeyer and Kalderon 1998, Preat 1992). Surprisingly, however, amorphic *Sufu* mutants alone are viable and without any obvious morphological change in *Drosophila*. Consistently, no ectopic Hh pathway activation was detected in $Sufu^{LP}$ clones (Methot and Basler 2000). One possible explanation for this paradox is that Ci, in this case, may still be subjected to another layer of negative regulation imposed by Cos2. Indeed, in *cos2* mutant background, removing Sufu results in ectopic activation of *en*, indicative of the formation of a hyperactive Ci that activates the highest level of Hh pathway activity (unpublished data). How does Sufu achieve its inhibition on Ci? Former yeast two hybrid data suggest that Sufu directly interacts with Ci (Monnier et al. 1998). This interaction is thought important for Sufu to inhibit Ci activity by sequestering it in the cytosol (Methot and Basler 2000). Nevertheless, recent study suggests that Sufu may also translocate to nuclei with Ci and inhibit the Ci activity likely through recruiting a transcriptional corepressor(s) (Sisson B. E. et al. 2006).

Interestingly, Cos2, Fu, and Sufu all undergo phosphorylation in response to Hh stimulation (Lum et al. 2003b); however, the kinases in charge of the phosphorylation have not been identified yet and the biological functions of these phosphorylation events are still unknown. A previous *in vitro* kinase assay revealed that Fu is capable to auto-phosphorylate itself (Nybakken et al. 2002). It was also observed that loss of Fu kinase activity results in a reduction of phosphorylation of both Sufu and Cos2 (Ho et al. 2005, Lum et al. 2003b). Particularly for Cos2, a kinase assay carried in a baculovirus system showed that Fu phosphorylates Cos2 at Ser572 and Ser931 (Nybakken et al. 2002). Combining all these findings, it is likely that Hh somehow can trigger the auto-phosphorylation and activation of Fu. Then active Fu can antagonize the activity of Sufu or Cos2 by either directly or indirectly phosphorylating them. Hence, further characterizing the Fu-mediated phosphorylation sites in Fu and its substrates will benefit our understanding of the mechanisms underlying the regulation of HSC by Hh.

5. The Ci Transcription factor

The only identified transcription factor for *Drosophila* Hh signaling pathway is Ci, a member of the Gli family of Zn finger transcription factors (Orenic et al. 1990, Ruiz i Altaba 1997). Full length Ci protein (aa 1-1397) contains a DNA-binding domain (aa 440-620) consisting of five Zn finger domains and a C-terminal transactivation domain (aa 1020-1160) essential for recruiting the coactivator CREB-Binding Protein (CBP) complex (Akimaru et al. 1997, Alexandre et al. 1996). In physiological condition, Ci exists in at least three forms: a truncated repressor form (Ci^R), a full length form (Ci^F), and a hyperactive liable form (Ci^A) (Aza-Blanc et al. 1997, Methot and Basler 1999, Ohlmeyer and Kalderon 1998). More accurately, Ci^F can be further defined in two different states, a cytosolic default form and a nuclear-localized partially active form (Ascano et al. 2002). The generation and balance of different forms of Ci is tightly controlled by several layers of regulatory processes, which assure Ci to precisely output different levels of Hh signaling activity.

In the absence of Hh, phosphorylating Ci^F by Cos2-recruited multiple kinases, including PKA, CK1 and GSK3, targets it to the Slimb-Cul1-based E3 ubiquitin ligase complex for ubiquitin/proteasome-mediated proteolysis to generate a truncated form, Ci^R. (Jia et al. 2002, Jia et al. 2005, Price and Kalderon 2002, Smelkinson et al. 2007, Zhang W. et al. 2005). Ci^R, lacking the transactivation domain but retaining the DNA-binding domain, enters nucleus and functions as a transcriptional repressor to turn off the expression of Hh target gene, such as *dpp* (Aza-Blanc et al. 1997, Methot and Basler 1999) (Fig. 1.1).

Low levels of Hh suffice to prevent Ci processing by dissociating Cos2-kinases complex from Ci (Zhang W. et al. 2005). Although accumulated Ci^F induces *dpp* expression, it is not fully activated given its failure to activate *ptc* and *en* expression (Methot and Basler 1999) (Fig. 1.1). It is thought that in this case Sufu may still inhibit Ci^F activity probably through cytoplasmic retention, recruiting a corepressor(s) in the nucleus or both (Chen C. H. et al. 1999, Cheng S. Y. and Bishop 2002, Methot and Basler 2000, Wang G. et al. 2000b).

In the presence of mid-to-high levels of Hh, Ci^F is converted into a hyperactive form, Ci^A, likely through releasing the Sufu-mediated repression (Methot and Basler 1999, Ohlmeyer and Kalderon 1998). Ci^A turns on Hh-responsive genes including *ptc* and *en* (Methot and Basler 1999, Ohlmeyer and Kalderon 1998), and becomes very unstable due to ubiquitination and degradation by a Hh-induced Math and BTB domain containing protein (HIB) E3 ligase complex (Kent et al. 2006, Zhang Q. et al. 2006) (Fig. 1.1).

Interestingly, all the above regulations can be accurately reminisced by the specific expression pattern of Ci as well as Hh-responsive genes in *Drosophila* wing discs, which makes the *Drosophila* wing disc an ideal model for studying the Hh pathway. As shown in Fig. 1.2B, Hh is restrictedly expressed in the posterior (P-) compartment cells of the wing disc, diffuses into anterior (A-) compartment and forms a gradient along the A/P boundary; while Ci is only expressed in the A-compartment cells (Fig. 1.2A). In A-compartment cells far away from the A/P boundary, because of the absence of Hh, Ci^F is processed into

the truncated repressor form that can not be detected by an anti-Ci antibody specifically recognizing the C-terminus of Ci, which thus results in the low level of Ci staining (Fig. 1.2A, region 1). In the zone near the A/P boundary where low levels of Hh are received, Ci processing is blocked as indicated by the elevation of full length Ci staining (Fig. 1.2A, region 2). *dpp* expression is turned on due to the lack of Ci^R and the accumulation of Ci^F (Fig. 1.2B). In the region just abutting the A/P boundary where high levels of Hh are received, Ci^F escapes from the inhibition of Sufu and matures to a hyperactive form Ci^A, which activates *dpp* as well as high threshold downstream genes such as *ptc* and *en* expression (Ohlmeyer and Kalderon 1998) (Fig. 1.2B). Of note, full length Ci staining becomes low again in this narrow strip of anterior cells (Fig. 1.2A, arrow), which is not due to the processing but rather a negative-feedback degradation mediated by the HIB E3 ligase complex (Kent et al. 2006, Ohlmeyer and Kalderon 1998, Zhang Q. et al. 2006).

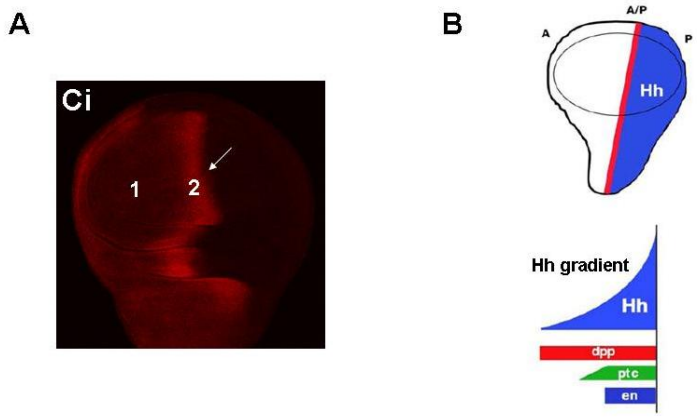


Figure 1.2 *Drosophila* wing disc as a model for studying Hh pathway. (A) A wild-type wing disc was immunostained to show the expression of Ci^F. Region 1 represents A-compartment cells far away from the A/P boundary. Region 2 represents A-compartment cells near the A/P boundary. Arrow indicates the region just abutting the A/P boundary. (B) A diagram shows the Hh gradiant and the expression pattern of Hh target genes. Low levels of Hh suffice to turn on *dpp*, whereas high levels of Hh are required to turn on *ptc* and *en*. See text for details.

Beyond the balance between activator and repressor forms of Ci, new evidence also shows that enhancers of Hh target genes can respond selectively to the activator and repressor forms of Ci, and that

this selectivity is determined by the affinity of Ci sites within those enhancers (Parker et al. 2011, Whitington et al. 2011), providing an additional layer of regulation controlling the output of Hh gradient by Ci.

6. Divergence between *Drosophila* and Vertebrate Hh pathway

Similar to other signaling pathways (such as Notch and Wnt), the major components of the Hh pathway undergo gene duplication during evolution (Ingham and McMahon 2001), implying that vertebrates may adopt a more complex system for the Hh signaling transduction. Indeed, recent studies reveal that, despite that the essential logic of the Hh signaling transduction is conserved across species, the detailed molecular mechanism of the Hh signaling transduction differs significantly between *Drosophila* and vertebrates (Huangfu and Anderson 2006, Varjosalo et al. 2006).

First, unlike *Drosophila* having one *hh* gene, vertebrates have multiple *hh* homologs. For example, mouse has three *hh* homologs, *Shh*, *Ihh* and *Dhh* (Ingham and McMahon 2001). They exhibit distinct expression patterns and play different roles in development. Shh and Ihh have been shown essential for embryonic development, whereas Dhh regulates spermatogenesis (Bitgood et al. 1996, Chiang et al. 1996, St-Jacques et al. 1999).

Then at the cell membrane, vertebrates adopt two *ptc* homologs (*ptch1&ptch2*) receiving the Hh signal and a single Smo protein transducing the Hh signaling to downstream (Goodrich et al. 1997, Wolff et al. 2003). Of note, the cytoplasmic C-tail is significantly different between *Drosophila* and vertebrate Smo, with only the 180 amino acid juxtamembrane region being highly related whereas the more C-terminal region showing short patches of homology (Huangfu and Anderson 2006). Another divergent region between *Drosophila* and vertebrate Smo is the N-terminal extracellular CRD. While the CRDs of fish and mouse Smo are 70% identical, there is only 43% identity between *Drosophila* and mouse CRDs (Huangfu and Anderson 2006). Mutations in *Drosophila* CRD disrupt Smo activity *in vivo* (Alcedo et al.

2000, Nakano et al. 2004), whereas deletion of the CRD in mammalian Smo does not affect its activity when overexpressed in certain cell lines (Huangfu and Anderson 2006, Murone et al. 1999, Taipale et al. 2002). In line with these divergences, recent studies have revealed that Smo is activated by distinguished mechanisms in *Drosophila* and vertebrates. 1) Most of the PKA/CK1 phosphorylated residues identified in the *Drosophila* Smo C-tail are not conserved in vertebrate Smo. Instead, mammalian Smo is activated through phosphorylation of a different set of sites in its C-tail by CK1α and GRK2 (Chen W. et al. 2004b, Chen Y. et al. 2011b). 2) In contrast to *Drosophila* Smo accumulating on the cell surface upon activation, the pathway activation leads to internalization of mammalian Smo, a process that involves β-arrestin2 (Chen W. et al. 2004b, Incardona et al. 2002). 3) Mammalian Smo requires to be recruited to a specialized cellular structure, cilia, for activation (Huangfu and Anderson 2006). 4) Several exogenous small molecules have been identified as either agonists or antagonists for mammalian Smo, consistent with a prevailed speculation that Ptc may transport an endogenous small molecule(s) to regulate the mammalian Smo activity. However, similar effects of these small molecules have not been seen in *Drosophila* (Chen J. K. et al. 2002, Chen W. et al. 2001, Taipale et al. 2002). Of note, albeit the above divergences, mammalian Smo proteins exist as constitutive dimers/oligomers similar to *Drosophila* Smo, within which the C-tails adopt a close conformation and apart from each other in the absence of Hh whereas undergo a conformational switch and cluster together upon Hh stimulation (Chen Y. et al. 2011b, Zhao et al. 2007a).

The most divergence between *Drosophila* and vertebrate Hh pathway exists at the level of the cytoplasmic signal transduction. *Drosophila* adopts Gαi and Fu/Cos2/Sufu signaling complex to relay signal from Smo to Ci (Lum et al. 2003b, Ogden et al. 2008, Robbins et al. 1997); however, the roles of vertebrate homolog of these components in transducing Hh signal are either controversial or insignificant. 1) Although early work in various vertebrate cell models indicated that Smo has the ability to at least couple with G-proteins (DeCamp et al. 2000, Riobo et al. 2006), whether this signaling mechanism is necessary in all cell types is not clear. For example, it has been shown that overexpression of a constitutively active Gαi protein did not significantly affect Hh-dependent neural cell specification in the

chick neural tube (Low et al. 2008); whereas a recent study shows that Hh-induced proliferation of cerebellar granular neuronal precursors requires the combined activity of Gα(i2) and Gα(i3) proteins (Barzi et al. 2011). 2) Although two orthologs of Cos2, Kif7 and Kif27, have been found sharing 39% and 37% sequence similarity with *Drosophila* Cos2 respectively (Huangfu and Anderson 2006, Katoh and Katoh 2004), early *in vitro* study in mouse cells led to the conclusion that neither protein has a role in Hh signaling. (Varjosalo et al. 2006). Consistent with this, the mouse Smo C-terminal domain corresponding to the region in *Drosophila* that is phosphorylated in response to Hh and binds to Cos2 is not required for mouse Smo function (Varjosalo et al. 2006). However, later, three independent groups demonstrated that Kif7 functions as a cilia-associated protein and is a critical regulator of Gli transcription factors in mammalian Hh signaling (Cheung et al. 2009, Endoh-Yamagami et al. 2009, Liem et al. 2009). In keeping with the conserved role of Cos2 family proteins in Hh signaling, one recent study shows that *Drosophila* Cos2 binds to Gli1 and can silence mammalian Gli1 in fly in a Hh-regulated manner, and that Cos2 and Kif7 can also direct Gli3 and Ci processing in fly (Marks and Kalderon 2011); while another study demonstrates that Kif7 plays a role in the turnover of Sufu and the exclusion of Sufu-Gli complexes from the primary cilium, and regulates the activity of Gli transcription factors through both Sufu-dependent and -independent mechanisms (Hsu et al. 2011). 3) As for the homolog of *fu*, *stk36*, morpholino knock down experiments indicated that it is required for responses to high levels of Hh in *zebrafish* (Wolff et al. 2003); however, it was striking to find that mice lacking Stk36 activity survive beyond birth and have no apparent defects in Hh signaling (Chen M. H. et al. 2005, Merchant et al. 2005), instead showing dysfunction in construction of the central pair apparatus of motile, 9+2 cilia (Wilson et al. 2009). It is thought that the function of Fu in the mammalian Hh pathway could be substituted by some other kinase(s). A Fu-related kinase, ULK3, has been reported as a positive regulator of Shh signaling in mammals (Maloverjan et al. 2010a, Maloverjan et al. 2010b). In addition, a Kinome siRNA screen identified cdc2l1 as another candidate kinase that is necessary and sufficient for activation of the mammalian Hh pathway (Evangelista et al. 2008). 4) Sufu mutant flies are viable and fertile, showing a phenotype only when *fu* is also mutated (Preat 1992). By contrast, removing Sufu in *zebrafish* or mouse

embryos produces strong gain of Hh signaling phenotypes even when Fu is present (Cooper et al. 2005, Wolff et al. 2003). In addition, humans heterozygous for *sufu* mutations have a predisposition to medulloblastoma, as seen with mutations in *Ptch1* (Pasca di Magliano and Hebrok 2003, Taylor et al. 2002). Thus, Sufu plays a crucial role in the negative regulation of the Hh pathway in vertebrates.

Then the question is how vertebrate Hh signaling is transduced given the above divergences of the cytoplasmic transducers. From recent studies, it turns out that vertebrates employ a special subcellular structure, cilia, to achieve this goal. It is known that intraflagellar transport (IFT) is a process required for the assembly and maintenance of cilia and flagella (Pazour et al. 2002). Several components essential for IFT, including three IFT complex B proteins (Ift172/Wimple, Polaris/Ift88/Ttc10, Ngd5/Ift52), an IFT anterograde motor subunit (Kif3a) and an IFT retrograde motor subunit (Dnchc2), have been shown crucial for the vertebrate Hh signaling at a step between Smo and Gli transcription factors (Huangfu and Anderson 2005, Liu A. et al. 2005, Nanba et al. 2003). By contrast, flies lacking homologs of Ift88, Ift172 and Kif3a do not have any defect in the Hh signaling transduction (Avidor-Reiss et al. 2004, Han Y. G. et al. 2003). The mechanism underlying the cilia-mediated Hh signaling transduction in vertebrates remains to be explored. Several evidences suggest that Smo and Glis require localization to cilia for their activity (Aanstad et al. 2009, Corbit et al. 2005, Huangfu and Anderson 2005, Liu A. et al. 2005, Wen et al. 2010). This signal-dependent localization in cilia is reminiscent of the Hh-induced cell-surface accumulation of Hh components in *Drosophila,* which raises an interesting possibility that cilia may act as signaling centers to concentrate Hh pathway components and activate pathway by enhancing their interactions (Huangfu and Anderson 2006). On the other hand, given the microtubule-association feature of IFT proteins (Katoh and Katoh 2004), it is also likely that IFT proteins may substitute for the function of Cos2 and provide the missing link between Smo and Gli proteins (Huangfu and Anderson 2005).

Finally, compared to *Drosophila* only having one Ci transcription factor, vertebrates use three Gli proteins (Gli1, Gli2, and Gli3) to output the Hh signal. Sequence analysis suggests that Gli proteins and

Ci have limited homology outside the Zn finger DNA binding domain, and further studies reveal that individual Gli proteins have specific biochemical properties and are subjected to different modes of post-transcriptional regulation. Gli1 can not be processed into a repressor form and only functions as a transcriptional activator (Dai et al. 1999, Lee J. et al. 1997). Although both Gli2 and Gli3 can be proteolytically processed, the dominant role of Gli2 is gene activation whereas Gli3 majorly plays a gene repression role (Aza-Blanc et al. 2000, Wang B. et al. 2000a). Remarkably, either activation of Gli2 or loss of Gli3 repression function results in an induction of Gli1 (Hu M. C. et al. 2006, Lee J. et al. 1997, Marigo et al. 1996, Motoyama et al. 2003), implying the evolution of a feedback loop in the vertebrate Hh transcriptional network. Several vertebrate proteins that modulate Gli transcriptional activity have been identified. For example, MIM/BEG4, a transcriptional target of mammalian Hh signaling, can associate with Gli1&Gli2 and potentiate their transcriptional activity (Callahan et al. 2004, Gonzalez-Quevedo et al. 2005); whereas Ski, normally functioning as a corepressor, can specifically bind Gli3 and regulate its repression activity (Dai et al. 2002). Furthermore, vertebrates utilize a set of different degradative pathways to control the limited or complete proteolysis of Gli proteins. For example, binding to β-TrCP, an E3 adapter, is required for the destruction of Gli1, either the processing or the destruction of Gli2, and the processing of Gli3 (Bhatia et al. 2006, Huntzicker et al. 2006, Pan et al. 2006); while additional degrons specifically present in Gli1 control its stability using the Numb-Itch ubiquitination pathway (Di Marcotullio et al. 2006, Huntzicker et al. 2006). Taken together, vertebrates employ a more complex Hh transcriptional network, which could provide the opportunity for additional modulation of the pathway.

7. Purpose of Study

My graduate study aims to investigate the mechanisms underlying the regulation of the Hh pathway. One big puzzle in the field is how the Hh signaling transduces from the membrane receptor system to the downstream transcription factor, Ci. In chapter two, I dissected this by asking three questions: 1) How does Hh-induced change of subcellular localization and conformational state of the

GPCR like protein Smo activate the cyptoplasmic Cos2/Fu signaling complex? 2) What is the molecular basis for the Fu kinase activation? 3) How does active Fu promote the transcriptional activity of Ci?

Then in the rest part of my study, I focused on the mechanisms regulating the activity of Ci. In chapter three, I intended to investigate the repression function of the N-terminus of Ci by mapping the minimum repression motif(s) as well as identifying possible corepressor factor(s). In chapter four, working with a former post-doc, Dr. Qing Zhang, we characterized multiple Ser/Thr rich motifs in Ci that sever as degrons for HIB/SPOP E3 complex. Finally, in chapter five, I identified a PY-NLS in the N-terminal conserved domain of Ci and explored how Ci activity is regulated by modulating its nucleo-cytoplasmic transportation.

CHAPTER II

Hedgehog-induced Smoothened conformational switch assembles a signaling complex that activates Fused by promoting its dimerization and phosphorylation

Introduction

How different thresholds of Hh morphogen specify distinct outcomes is still poorly understood. In *Drosophila*, Hh binding to Ptc abrogates its inhibition on Smo and induces extensive phosphorylation of the Smo cytoplasmic C-tail by PKA, CK1, GSK3, Gprk2 and CK2 (Apionishev et al. 2005, Chen Y. et al. 2010, Denef et al. 2000, Jia et al. 2010, Jia et al. 2004, Zhang C. et al. 2004). This phosphorylation disrupts Arg-mediated intramolecular electrostatic interactions in Smo C-tail, leading to Smo activation by inducing a conformational switch and dimerization of Smo C-tails (Zhao et al. 2007a). Interestedly, the degree of Smo conformational change and the level of Smo activity correlate with the level of Smo phosphorylation, which in turn may correlate with the level of Hh. Thus, Smo acts as a rheostat to transduce graded Hh signal through differential phosphorylation (Zhao et al. 2007a). However, how Smo conformational change activates the intracellular signaling components remains elusive.

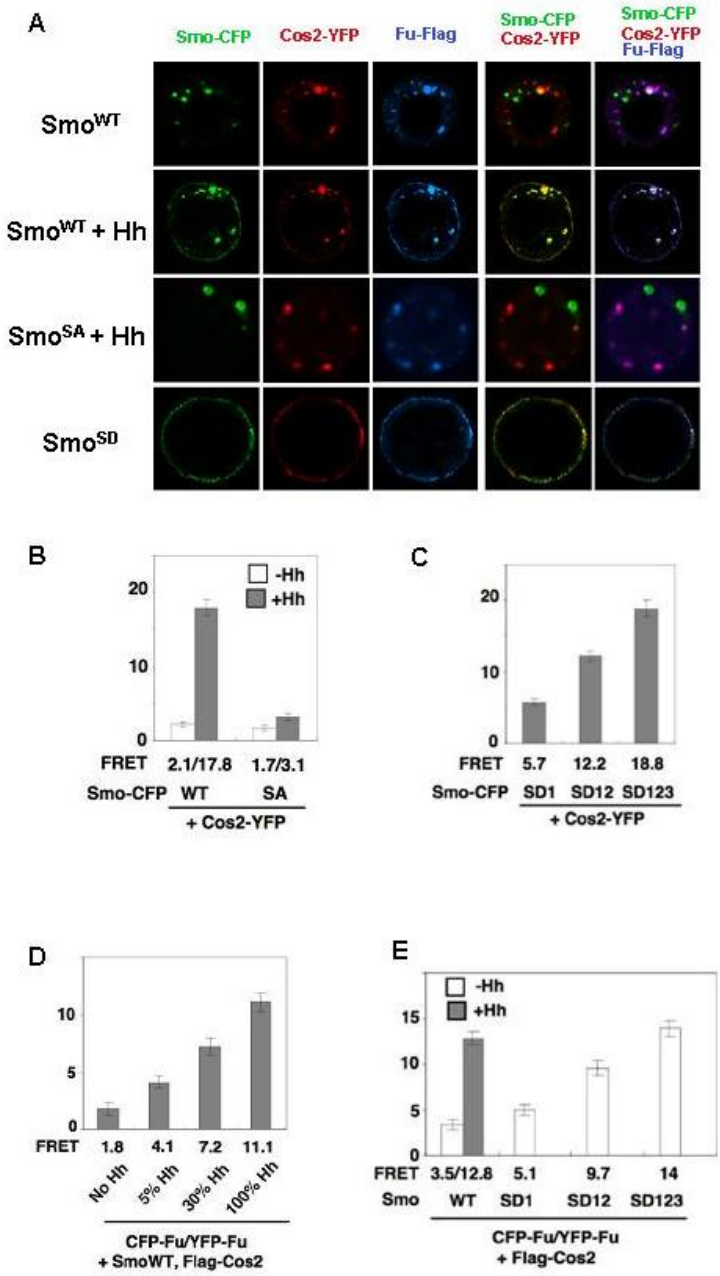
Several studies have demonstrated that the cytoplasmic C-tail of Smo that interacts with Cos2/Fu cytoplasmic signaling complex as well as activates Gαi is required for Smo to transduce Hh signal to downstream (Jia et al. 2003, Lum et al. 2003b, Ogden et al. 2003, Ogden et al. 2008). Interaction between Smo and Cos2/Fu is mediated by at least two regions in Smo C-tail: a membrane proximal domain (aa 651-686) and a C-terminal domain (aa 818-1035) (Jia et al. 2003, Lum et al. 2003b). While the C-terminal Cos2/Fu binding domain is essential for Smo activity (Jia et al. 2003), the membrane proximal Cos2-binding domain mediates an inhibition of Smo phosphorylation by Cos2/PP4, which can be

alleviated by Fu kinase activity (Claret et al. 2007, Jia et al. 2009, Liu Y. et al. 2007). Because the association between Smo and Cos2/Fu is observed even in the absence of Hh, complex formation per se is insufficient for pathway activation. It is thus likely that Smo and Cos2/Fu may form distinct complexes depending on the status of Hh signaling, and that pathway activation may rely on changes in the location, composition and conformational state of the complexes.

In *Drosophila*, Fu is a critical component of the intracellular Hh signaling complex, since mutations of *fu* result in a loss of Hh signaling (Alves et al. 1998). *Drosophila* Fu consists of an N-terminal serine/threonine kinase domain (aa 1-305) and a C-terminal regulatory domain (aa 306-805) (Therond P. et al. 1996a). Using the yeast two-hybrid method and *in vitro* binding assay, a Sufu-interaction domain (aa 306-436) and a Cos2-interaction domain (aa 523-805) were mapped to the C-terminal region of Fu (Monnier et al. 1998, Robbins et al. 1997). So far, the direct substrate(s) of Fu has not been identified, although it has been shown that mutations of either Cos2 or Sufu can suppress the *fu* phenotype and Fu is required for Hh-induced phosphorylation of Cos2 and Sufu (Dussillol-Godar et al. 2006, Lum et al. 2003b, Nybakken et al. 2002, Ruel et al. 2007). Besides, Fu itself also undergo phosphorylation in response to Hh in both *Drosophila* embryos and cultures of S2 cells (Lum et al. 2003b, Therond P. P. et al. 1996b), but the biological function of Fu phosphorylation and the mechanisms by which Fu activity is regulated by Hh are still unknown.

Studies on *fuII* alleles suggest that the Cos2-interaction domain in Fu C-tail is essential for its activity (Robbins et al. 1997, Therond P. et al. 1996a), which raises an interesting possibility that Fu needs being recruited to Smo C-tail by Cos2 for activation. Nevertheless, simply recruiting Cos2/Fu complex by a membrane-tethered form of Smo C-tail does not fully activate the Hh pathway (Jia et al. 2003, Zhao et al. 2007a), implying that additional regulation needs being imposed for Fu activion. Interestingly, previous study in our lab showed that dimerized Smo C-tails induce Fu phosphorylation similar to Hh stimulation (Zhao et al. 2007a). Furthermore, by co-localization and FRET analysis both *in*

vitro and *in vivo*, former lab members found that Hh stimulation or Smo phosphorylation promotes the assembly of Smo-Cos2-Fu complexes as well as increases Fu-Fu intermolecular interactions (Fig. 2.1). Combining all these observations, we speculate that Hh-induced Smo conformational change and clustering of Smo cytoplasmic tails may activate Ci by inducing Fu dimerization through Cos2, leading to Fu phosphorylation and activation.



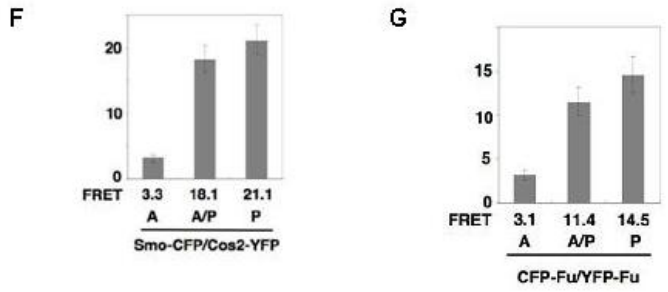


Figure 2.1 Hh stimulation or Smo phosphorylation enhances Smo-Cos2 and Fu-Fu interactions (Former results). (A) S2 cells transfected with indicated constructs with or without Hh treatment were immunostained to show the expression of different forms of Smo-CFP (green), Cos2-YFP (red) and Fu-Flag (blue). Columns 4 and 5 are the merged images of CFP/YFP or CFP/YFP/Flag signals, respectively. Row 1: In the absence of Hh, Cos2 co-localized with Fu, but Cos2/Fu complex barely co-localized with Smo. Row 2: Hh treatment dramatically increased the co-localization between Smo and Cos2/Fu complex. Row 3: A phospho-deficient form of Smo, Smo^{SA} (Jia et al. 2004), abolished the Hh-induced co-localization between Smo and Cos2/Fu complex. Row 4: A phospho-mimetic constitiutively active form of Smo, Smo^{SD} (Jia et al. 2004), co-localized with Cos2/Fu complex in the absence of Hh stimulation. (B) FRET efficiency between CFP-tagged wild-type Smo or Smo^{SA} and Cos2-YFP in S2 cells cotransfected with Myc-Fu, and treated with or without Hh (mean ± s.d., n≥10). Hh stimulation enhanced the FRET between Smo-CFP/Cos2-YFP, but not that between Smo^{SA}-CFP/Cos2-YFP. (C) FRET efficiency between CFP-tagged wild-type Smo or Smo^{SD} variants in S2 cells cotranfected with Myc-Fu. Gradually increasing the phospho-mimetic mutations of Smo progressively increased the FRET between Smo-CFP/Cos2-YFP. (D) FRET efficiency between CFP-Fu and YFP-Fu cotransfected with Flag-Cos2 into S2 cells stably expressing Myc-Smo and treated without or with 5%, 30% and 100% of the Hh-conditioned medium (mean ± s.d., n≥10). Hh gradient progressively increased the FRET between CFP-Fu/YFP-Fu. (E) FRET efficiency between CFP-Fu and YFP-Fu in S2 cells cotransfected with Flag-Cos2 and Smo phospho-mimectic variants. Gradually increasing the phospho-mimectic mutations of Smo progressively increased the FRET between CFP-Fu/YFP-Fu. (F-G) FRET efficiency between Smo-CFP/Cos2-YFP (F) or CFP-Fu/YFP-Fu (G) expressed in wing discs using *MS1096* at 25 ^{0}C (mean ± s.d., n≥5). A: A-compartment >30 cells from the A/P boundary; P: P-compartment cells; A/P: A-compartment 1-10 cells from the A/P boundary.

In this study, we investigate how Hh signaling alters the state of Smo-Cos2-Fu complex to activate Fu and how activated Fu leads to a change in Ci activity. We demostrate that Hh-induced Smo conformational switch and clustering of Smo C-tails assemble active Smo-Cos2-Fu signaling complexes that activate Fu by inducing its dimerization. We also found that Fu kinase dimerization or Hh stimulation induces multiple-site phosphorylation of its AL, and that phopho-mimetic mutations of Fu AL suffice to activate the Hh pathway. Finally, we provide evidence that active Fu regulates Hh transcription factor, Ci, by inhibiting its proteolysis into a repressor form and by promoting its transcriptional activator activity through triggering phosphorylation of Ci, Sufu and Cos2, and interfering with Ci-Sufu and Ci-Cos2-kinase complex formation.

Results

Forced Fu dimerization activates the Hh pathway

Dimerization/oligomerization triggers kinase activation in many cases (Pike et al. 2008). Thus we propose that Hh-induced dimerization may convert Fu kinase into an active form to turn on the Hh pathway. To test this possibility, we fused the GCN4 coiled-coil dimeriation motif (refereed as CC) or its mutant version (CCm) to the N-terminus of Fu, with the CCm as a dimerization-deficient control (Fig. 2.2A) (O'Shea et al. 1991). We established a *ptc-luc* reporter assay based on the premise that activated Fu releases the inhibition of Ci by Sufu (Ohlmeyer and Kalderon 1998). As shown in Fig. 2.2B, expression of Ci in S2 cells activated the *ptc-luc* reporter gene, which was suppressed by coexpression of Sufu (Fig. 2.2B, columns 1-3). Coexpression of a wild-type Fu or CCm-Fu did not release the inhibition of Ci by Sufu (Fig. 2.2B, columns 4 and 6). By contrast, CC-Fu derepressed Ci similarly to a phospho-mimetic constitutively active form of Smo, SmoSD123 (Fig. 2.2B, columns 5 and 8). Moreover, fusion of CC to a kinase-dead Fu variant (Fu^{G13V}) (Liu Y. et al. 2007) failed to derepress Ci (Fig. 2.2B, column 7), indicating that dimerization activates Fu depending on its kinase activity.

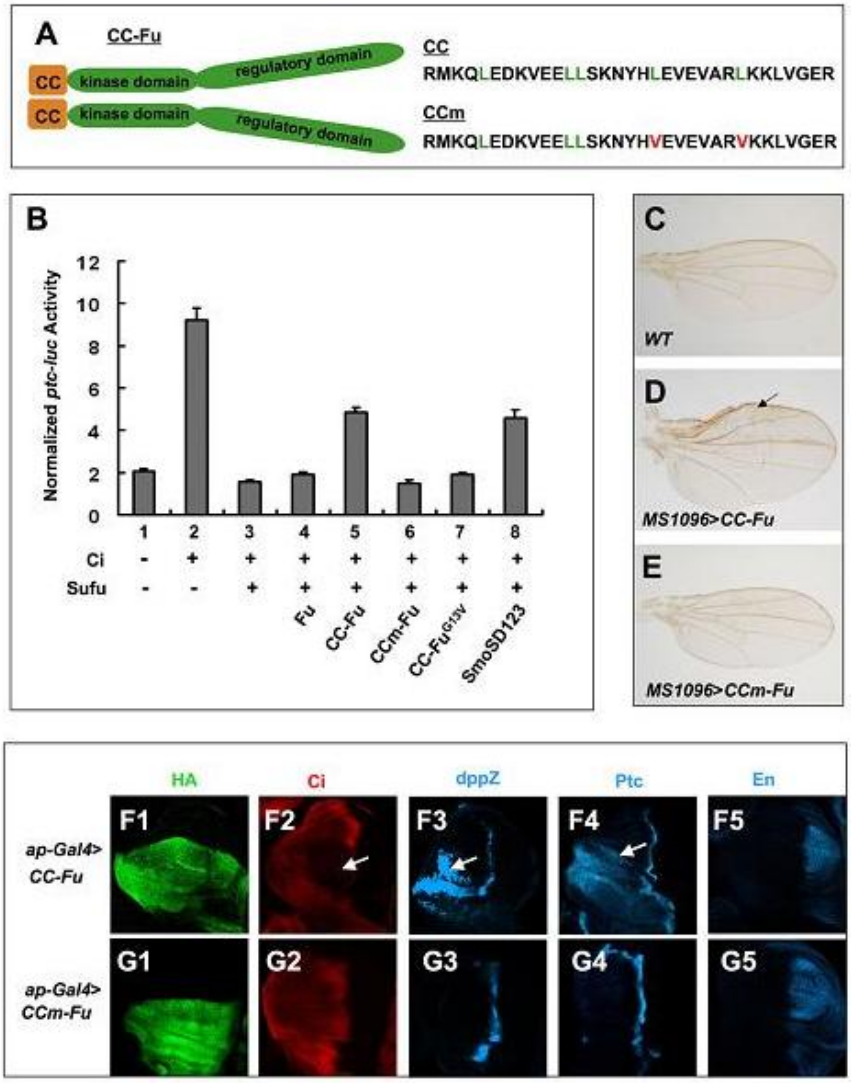


Figure 2.2 Fu kinase domain dimerization activates the Hh pathway. (A) Left: A diagram of dimerized CC-Fu. Right: Amino acid sequence of CC and CCm with green letters indicating the Leu residues essential for dimerization and red letters indicating Leu to Val substitutions that demolish the dimerization. (B) A *ptc-luc* reporter assay in S2 cells for the ability of Fu variants and SmoSD123 to release the inhibition of Ci by Sufu. The *y*-axis represents normalized *ptc-luc* activity. (C-E) A wild-type male wing (C) and male wings expressing *UAS-HA-CC-Fu* (D) or *UAS-HA-CCm-Fu* (E) with *MS1096* at 25 ^{0}C. Arrow in D indicates anterior overgrowth caused by expressing CC-Fu. (F1-G5) Wing discs expressing *UAS-HA-CC-Fu* (F1-F5) or *UAS-HA-CCm-Fu* (G1-G5) with *ap-Gal4* were immunostained to show the expression of HA, Ci, dpp-LacZ, Ptc and En. Expression of CC-Fu reduced the level of Ci, and induced ectopic expression of dpp-LacZ and Ptc (arrows in F2-4).

To determine whether dimerization of Fu activates the Hh pathway *in vivo*, *UAS* transgenes expressing either CC-Fu or CCm-Fu were introduced into the 75B1 locus using the *phiC31* integration system to ensure similar levels of transgene expression (Bischof et al. 2007). Expression of CC-Fu but not CCm-Fu using *MS1096* resulted in anterior overgrowth of adult wings, which is indicative of Hh pathway activation (Fig. 2.2C-E). When expressed using a dorsal compartment specific Gal4 driver *ap-Gal4*, CC-Fu but not CCm-Fu induced ectopic expression of Hh target genes including *dpp* and *ptc* in anterodorsal wing disc cells (Fig. 2.2F3-F4, G3-G4). We also noticed that CC-Fu but not CCm-Fu dramatically reduced the level of Ci^F (Fig. 2.2F2, G2), suggesting that CC-Fu may covert Ci^F into labile Ci^A. Consistent with activated Fu converting Ci^F into Ci^A by antagonizing Sufu (Ohlmeyer and Kalderon 1998), we found that coexpression of Sufu with CC-Fu restored Ci level and attenuated the ectopic *ptc* expression (data not shown).

Dimerized Fu activates Ci independent of Smo and Cos2

Although CC-Fu can activate Hh target genes, it failed to induce ectopic expression of *en* (Fig. 2.2F5) that requires high levels of Hh. One possibility is that CC-Fu may not effectively block Ci processing (see below) so that not enough Ci^F is available for conversion into sufficient amount of Ci^A required for *en* activation. To test this hypothesis, we coexpressed a processing-deficient form of Ci (Ci^U) (Methot and Basler 1999) with CC-Fu to boost the supply of Ci^F. Misexpression of Ci^U alone did not induce ectopic expression of Hh target genes in A-compartment cells away from the A/P boundary (Fig. 2.3A-A") (Methot and Basler 1999, Wang G. et al. 1999). Coexpression of CC-Fu but not CCm-Fu with Ci^U induced ectopic expression of both *ptc* and *en*, and promoted nuclear localization of Ci^U in A-compartment cells (Fig. 2.3B-C"; data not shown), indicating that Ci^U was converted into Ci^A by CC-Fu.

If Smo activates Fu by inducing its dimerization, one would predict that CC-Fu should activate Ci in the absence of Smo. Indeed, anteriorly situated *smo* mutant cells expressing CC-Fu and Ci^U still activated *en* (Fig. 2.3D-D"), suggesting that dimerization of Fu can activate Ci independent of Smo.

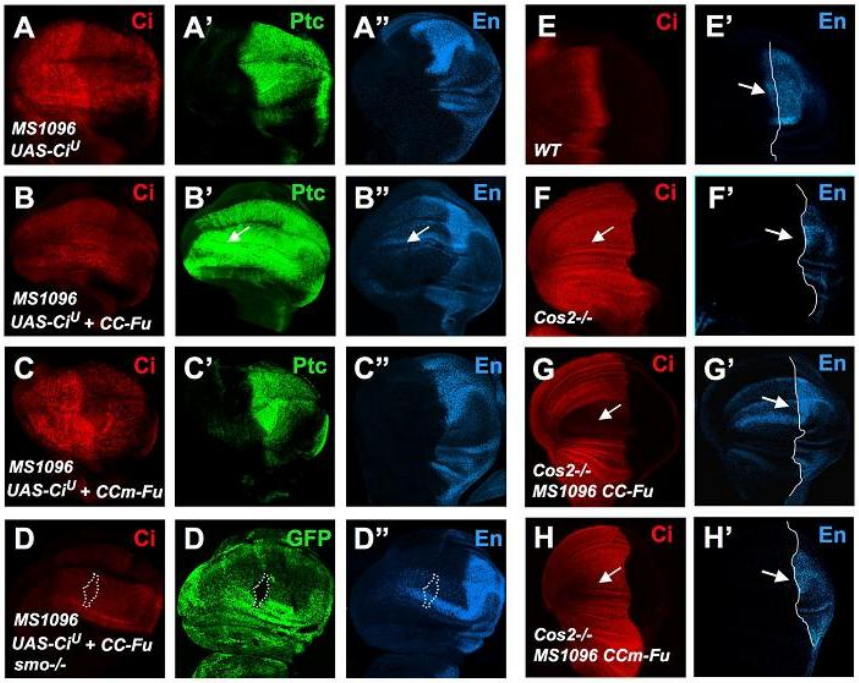


Figure 2.3 CC-Fu activates Ci independent of Smo and Cos2. (A-C") Wing discs expressing *UAS-Ci*^{U} alone (A-A"), or together with *UAS-HA-CC-Fu* (B-B") or *UAS-HA-CCm-Fu* (C-C") using *MS1096* were immunostained to show the expression of Ci, Ptc and En. (D-D") A wing disc carrying *smo*^{3} mutant clones (marked by the lack of GFP) and expressing Ci^{U} and CC-Fu with *MS1096* was immunostained to show the expression of Ci, GFP and En. Anteriorly situated *smo*^{3} mutant cells expressing CC-Fu and Ci^{U} induced ectopic *en* expression (outlined by dashed lines). (E-H') A wild-type wing disc (E, E') and *cos2*^{2} mutant wing discs without (F, F'), or with *UAS-HA-CC-Fu* (G, G') or *UAS-HA-CCm-Fu* (H, H') expressed by *MS1096* were immunostained to show the expression of Ci and En.

Cos2 is required for high levels of Hh signaling because Hh-dependent *en* expression is lost in *cos2* mutant discs (arrow in Fig. 2.3F') (Wang G. et al. 2000b). If the positive role of Cos2 is due to its requirement for Hh-induced Fu dimerization, one would expect that dimerized Fu should activate Ci in the absence of Cos2. Indeed, expressing CC-Fu but not CCm-Fu in *cos2* mutant wing discs rescued the anterior *en* expression near the A/P boundary (arrows in Fig. 2.3G', H') and induced ectopic *en* expression in A-compartment cells distant from the A/P boundary (Fig. 2.3G'). In addition, CC-Fu

markedly reduced the level of Ci^F normally accumulated in *cos2* mutant discs (arrows in Fig. 2.3F-H), consistent with CC-Fu converting Ci^F into labile Ci^A.

Dimerization and Hh signaling induce Fu activation loop (AL) phosphorylation

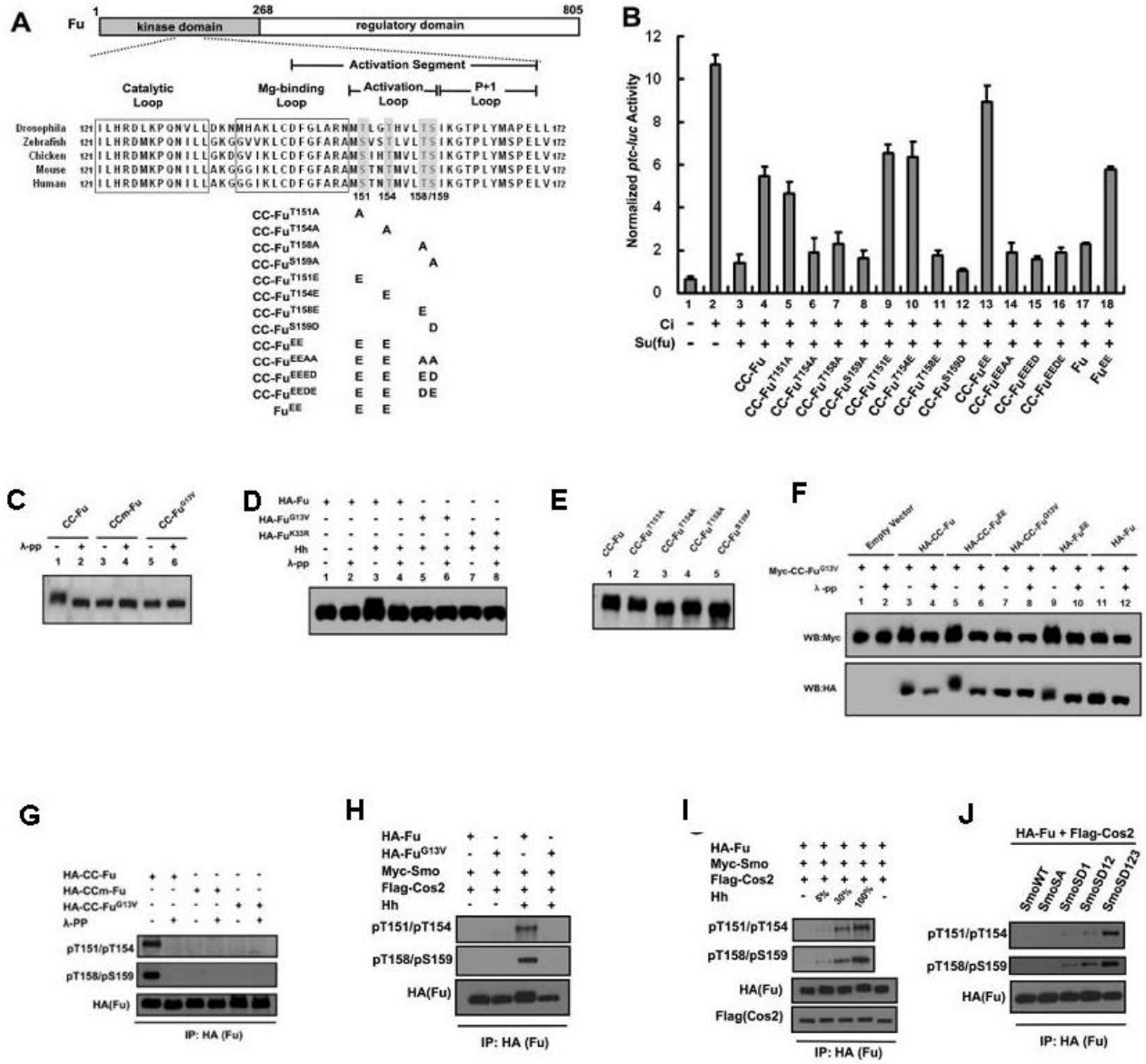


Figure 2.4 Dimerization and Hh signaling induce Fu AL phosphorylation (Results in G~J were generated by Dr. Shuang Li.). (A) A schematic drawing of Fu with a structure-based sequence alignment of the activation segments of Fu proteins from different species. Gray bars indicate the conserved Ser/Thr residues in Fu AL. Amino acid substitutions for CC-Fu and Fu variants are listed. (B) A *ptc-luc* reporter assay in S2 cells for the ability of indicated Fu variants to derepress Ci. (C) S2 cells were transfected with Myc-CC-Fu, Myc-CCm-Fu, and Myc-CC-Fu^{G13V}. Cell extracts without or with λ-protein phosphotase (λ-pp) treatment were run on a 6% (29:1 acrylamide:Bis-acrylamide) SDS-PAGE gel at 90V followed by western blot with anti-Myc antibody. CC-Fu but not CCm-Fu or CC-Fu^{G13V} exhibited a mobility shift, which was abolished by phosphatase treatment. (D) S2 cells were transfected with Myc-Smo, Flag-Cos2 and indicated constructs and treated with Hh-conditioned or control medium. Cell extracts without or with λ-pp treatment were run under the same condition as described in (C) followed by western blot with anti-HA antibody. Hh induced a mobility shift of HA-Fu but not of HA-Fu^{G13V} or HA-Fu^{K33R}. (E) S2 cells were transfected with Myc-CC-Fu and its derivatives with AL Ser/Thr mutated to Ala individually. Cells extracts were run under the same condition as described in (C) followed by western blot with anti-Myc antibody to show the effect of Ala mutations on the phosphorylation of CC-Fu. (F) S2 cells were transfected with the kinase-dead Myc-CC-Fu^{G13V} and different HA-tagged Fu variants. Cells extracts without or with λ-pp treatment were run under the same condition as described in (C) followed by western blot with anti-Myc antibody (Top panel) or anti-HA antibody (Bottom panel). (G) HA-tagged CC-Fu, CCm-Fu and CC-Fu^{G13V} were transfected into S2 cells. Cell extracts were immunoprecipitated with an anti-HA antibody, treated without or with λ-pp and followed by western blot using antibodies against pT151/pT154, pT158/pS159 or HA. (H) S2 cells transfected with HA-Fu or HA-Fu^{G13V} together with Myc-Smo and Flag-Cos2 were treated without or with Hh-conditioned medium. Cell extracts were immunoprecipitated with an anti-HA antibody, followed by western blot with the indicated antibodies. (I, J) S2 cells were transfected with the indicated constructs and treated without or with increasing levels of Hh. Cell extracts were immunoprecipitated with an anti-HA antibody, followed by western blot with the indicated antibodies.

Dimerization-induced auto-phosphorylation is one of the common regulatory mechanisms for kinase activation (Pike et al. 2008). It is currently assumed that unphosphorylated kinase transiently adopts an active conformation, which results in *trans*-phosphorylation of the neighboring kinase

molecules. Dimerization-induced high local concentration would increase the probability of autoactivation (Pike et al. 2008). Intriguingly, clustering of Smo C-tails or Hh stimulation leads to Fu phosphorylation (Therond P. P. et al. 1996b, Zhao et al. 2007a); thus, we next determined whether CC-mediated dimerization could induce Fu phosphorylation in a similar manner. When expressed in S2 cells, CC-Fu but not CCm-Fu or CC-Fu^{G13V} exhibited a mobility shift that could be abolished by phosphatase treatment (Fig. 2.4C), suggesting that Fu phosphorylation is induced by dimerization in a manner depending on its own kinase activity. In addition, Hh induced a mobility shift of wild-type Fu (HA-Fu) but not of two kinase-dead Fu variants (HA-Fu^{G13V} and HA-Fu^{K33R}; Fig. 2.4D), suggesting that Hh-induced Fu phosphorylation also depends on Fu kinase activity. Moreover, CC-Fu induced a mobility shift of a kinase-dead form of Fu when coexpressed in S2 cells (Fig. 2.4F), demonstrating that activated Fu can *trans*-phosphorylate another Fu molecule.

Because many kinases are activated by dimerization-induced auto-phosphorylation of their AL residues (Nolen et al. 2004, Pike et al. 2008), we then examined whether phosphorylation of any of the four conserved Ser/Thr residues (T151, T154, T158 and S159) in Fu AL is required for Fu activation in response to dimerization or Hh stimulation (Fig. 2.4A). We first mutated individual Ser/Thr residue to Ala (A) in CC-Fu (Fig. 2.4A), and found that T154A, T158A, and S159A mutations nearly abolished whereas T151A slightly reduced CC-Fu activity in a *ptc-luc* reporter assay (Fig. 2.4B, columns 5-8). Consistently, T154A, T158A, and S159A mutations nearly abolished whereas T151A slightly reduced CC-Fu mobility shift (Fig. 2.4E), suggesting that T154, T158, and S159 are critical for dimerization-induced Fu phosphorylation and activation. We also substituted the AL Ser/Thr residues individually or in combination to acid residue Glu (E) or Asp (D) to mimic phosphorylation (Fig. 2.4A). T151E or T154E slightly enhanced the activity of CC-Fu whereas T151E/T154E (CC-Fu^{EE}) resulted in a more dramatic enhancement (Fig. 2.4B, columns 9, 10 and 13), suggesting that phosphorylation at T151 and T154 promotes Fu activation. However, T158E or S159D abolished the activity of CC-Fu (Fig. 2.4B, columns 11-12). Moreover, mutating these two residues either to A or D/E in the context of CC-Fu^{EE} also

abolished Fu activity (Fig 2.4B, columns 14-16), suggesting that the function of T158 and S159 cannot be fulfilled by substitution with acidic residues.

To monitor Fu AL phosphorylation, we generated antibodies that specifically recognize phosphorylated T151/T154 (referred to as pT151/pT154) and T158/S159 (referred to as pT158/pS159) (see Materials and Methods). Using these antibodies, Dr. Shuang Li, my partner in this project, performed a series of western blot analysis. He found that both pT151/pT154 and pT158/pS159 antibodies detected CC-Fu but not CCm-Fu expressed in S2 cells and both signals were abolished by phosphatase treatment (Fig. 2.4G). In addition, pT151/pT154 did not detect CC-Fu^{T151A} and CC-Fu^{T154A} whereas pT158/pS159 did not detect CC-Fu^{T158A} and CC-Fu^{S159A} (data not shown), confirming the specificity of these antibodies. Furthermore, dimerization of the kinase-dead Fu (CC-Fu^{G13V}) failed to induce pT151/pT154 or pT158/pS159 signal (Fig. 2.4G), in line with the notion that dimerization-induced Fu AL phosphorylation depends on its kinase activity. Similarly, Hh stimulation induced phosphorylation at T151/T154 and T158/S159 of HA-Fu but not of HA-Fu^{G13V} (Fig. 2.4H), suggesting that Hh-stimulated phosphorylation of Fu AL also depends on Fu kinase activity. Interestingly, treatment with increasing levels of Hh or cotransfection with different phospho-mimetic forms of Smo resulted in a progressive increase in the levels of pT151/pT154 and pT158/pS159 signals (Fig. 2.4I, J), suggesting that Hh signaling may induce Fu AL phosphorylation in a dose dependent manner.

Fu AL phosphorylation triggers Hh pathway activation

To determine whether AL phosphorylation suffices to trigger Fu activation, we substituted T151 and T154 with E in the context of HA-Fu (HA-Fu^{EE}) (Fig. 2.4A). In a *ptc-luc* reporter assay, HA-Fu^{EE} released the inhibition of Ci by Sufu (Fig. 2.4B, column 18), albeit less effectively than HA-CC-Fu^{EE}. We also derived transformants carrying *UAS-HA-Fu*, *UAS-HA-Fu^{EE}* or *UAS-HA-CC-Fu^{EE}* inserted at the 75B1 locus using the *phiC31* integration system, and expressed these transgenes using the wing specific Gal4 driver *MS1096*. Expression of HA-Fu did not ectopically activate any Hh target genes and produced

apparently normal adult wings (Fig. 2.5A, I). Under the same condition, HA-CC-Fu induced weak ectopic expression of *dpp-lacZ* and *ptc* but failed to induce ectopic *en* expression (Fig. 2.5C) and produced adult wings with mild anterior overgrowth (Fig. 2.5J, 2.2D). In contrast, both HA-Fu^{EE} and HA-CC-Fu^{EE} induced strong ectopic expression of *dpp-lacZ* and *ptc* as well as ectopic *en* expression, which is more evident in the dorsal compartment where *MS1096* exhibits higher levels of expression (Fig. 2.5B, D). Females expressing HA-Fu^{EE} or HA-CC-Fu^{EE} exhibited severe overgrowth of their anterior wings (Fig. 2.5K-L) while males expressing these two forms of Fu were lethal because *MS1096* is located on the X chromosome and is expressed at higher levels in males than in females due to dosage compensation. These results demonstrate that the T151E/T154E mutation activates Fu and Hh pathway.

Although the activities of HA-Fu^{EE} and HA-CC-Fu^{EE} were nearly indistinguishable when expressed at high levels, they induced different levels of Hh pathway activation when expressed at lower levels. For example, when grown at 18^{0}C to reduce the activity of Gal4 and thus the expression of *UAS* transgenes, *MS1096>HA-Fu^{EE}* induced little if any ectopic expression of *dpp-lacZ* and *ptc* in female wing discs (Fig. 2.5M'-M") and induced weak ectopic expression of *dpp-lacZ* and *ptc* in male wing discs (Fig. 2.5N-N'). In contrast, *MS1096>HA-CC-Fu^{EE}* induced strong ectopic expression of *dpp-lacZ* and *ptc* in both female and male wing discs (Fig. 2.5O'-P'). Under these conditions, *MS1096>Fu^{EE}* females produced nearly normal wings (Fig. 2.5M) whereas *MS1096>CC-Fu^{EE}* female wings exhibited severe anterior overgrowth (Fig. 2.5O). Similar results were obtained by using a weak Gal4 driver, *C765* (Chen Y. et al. 2010), to express Fu^{EE} and CC-Fu^{EE} (data not shown). Furthermore, CC-Fu^{EE} exhibited more robust phosphorylation at T158/S159 than Fu^{EE} in S2 cells (data not shown), which is consistent with its being more active.

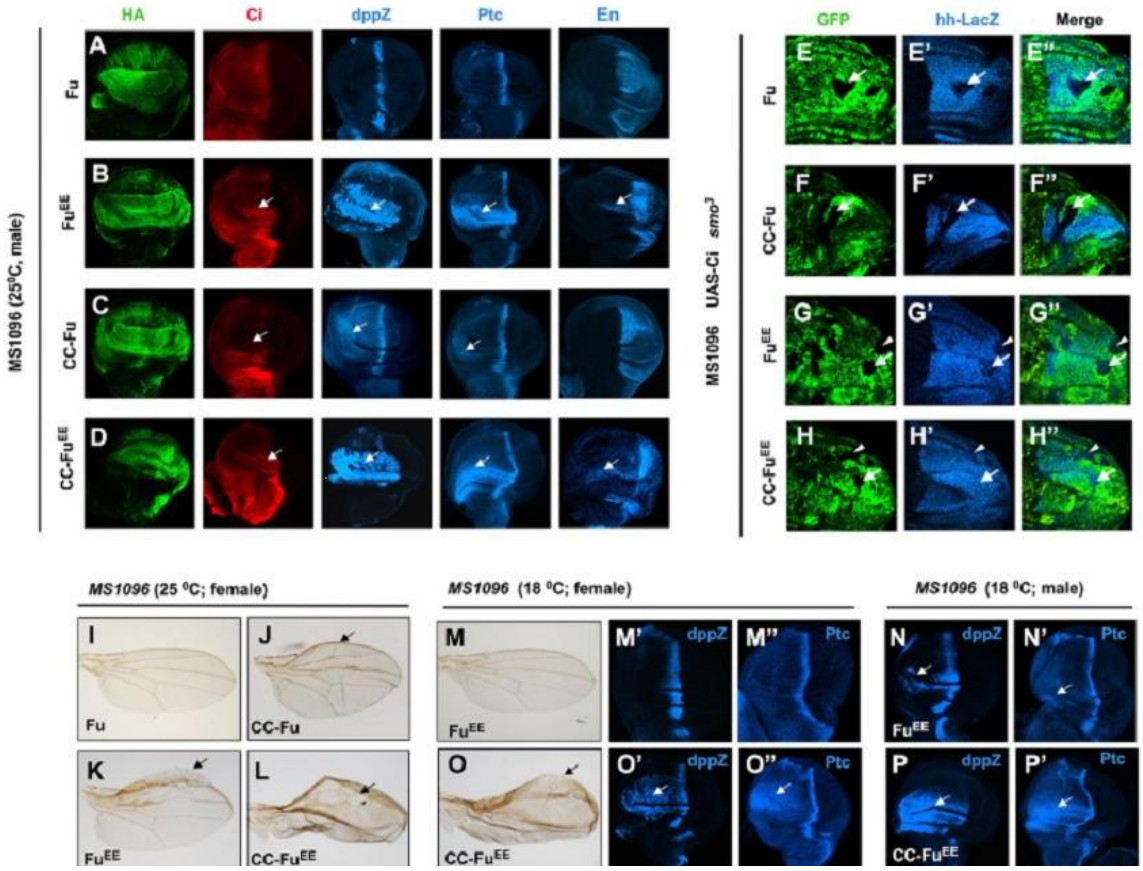


Figure 2.5 Fu AL phosphorylation activates Hh pathway. (A-D) Wing discs from males expressing *UAS-HA-Fu* (A), *UAS-HA-Fu*^{EE} (B), *UAS-HA-CC-F*u (C), and *UAS-HA-CC-Fu*^{EE} (D) with *MS1096* at 25^{0}C were immunostained to show the expression of HA (green), Ci (red), *dpp-LacZ*, Ptc or En (blue). Arrows indicate the effect of active forms of Fu on the expression of Ci and Hh target genes. (E-H") Wing discs carrying *smo*^{3} mutant clones and expressing *UAS-Ci* together with *UAS-HA-Fu* (E-E"), *UAS-HA-CC-Fu* (F-F"), *UAS-HA-Fu*^{EE} (G-G") or *UAS-HA-CC-Fu*^{EE} (H-H") using *MS1096* at 25^{0}C were immunostained to show the expression of GFP (green) and *Hh-LacZ* (blue). *smo*^{3} mutant clones are marked by the lack of GFP. Arrows indicate dorsal clones, and arrowheads indicate ventral clones. (I-L) Adult wings from females expressing *UAS-HA-Fu* (I), *UAS-HA-CC-Fu* (J), *UAS-HA-Fu*^{EE} (K) or *UAS-HA-CC-Fu*^{EE} (L) using *MS1096* at 25^{0}C. Arrows indicate anterior overgrowth. (M, O) Adult wings from females expressing *UAS-HA-Fu*^{EE} (M) or *UAS-HA-CC-Fu*^{EE} (O) using *MS1096* at 18^{0}C. Arrow in O indicates anterior overgrowth. (M'-P') Wing discs from females (M'-M", O'-O") or males (N-N', P-P') expressing *UAS-HA-Fu*^{EE} (M'-M", N-N') or *UAS-HA-CC-Fu*^{EE} (O',-O", P-P') using *MS1096* at 18 ^{0}C were immunostained to show the expression of *dpp-LacZ* (M', O', N, P) or Ptc (M", O", N', P'). Arrows indicate ectopic expression of *dpp-LacZ* or Ptc.

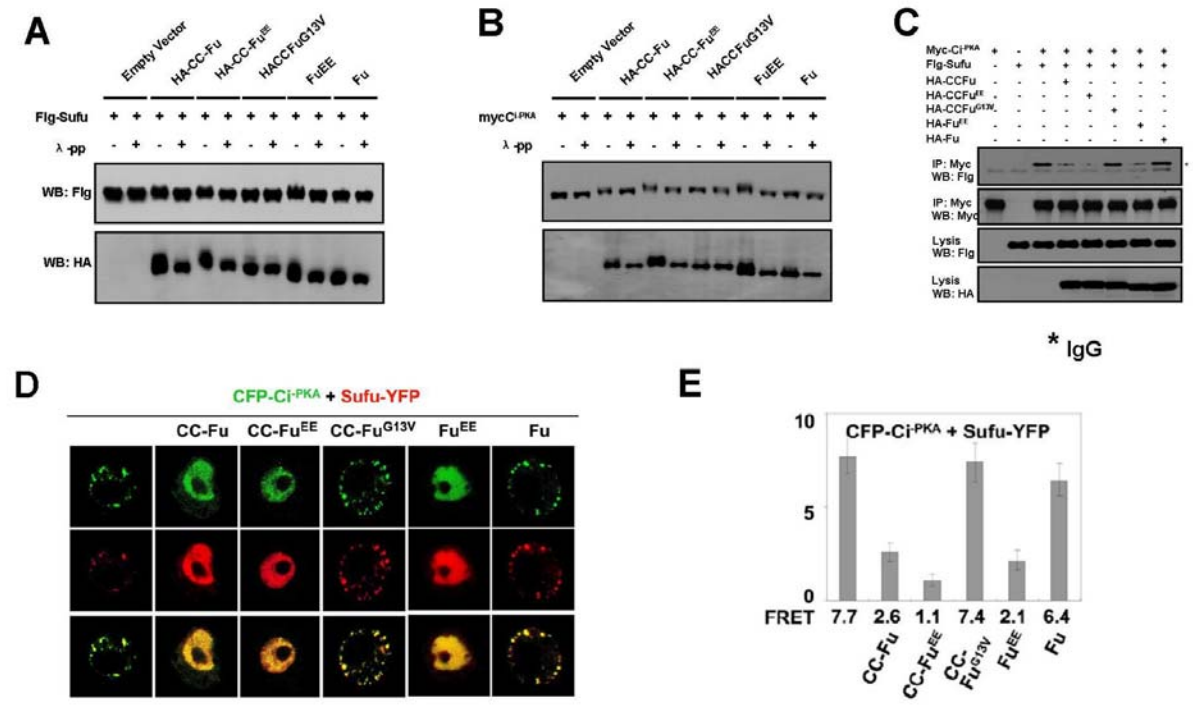
Activated Fu inhibits Ci^R production

We noticed that wing discs expressing *MS1096>Fu^{EE}* or *MS1096>CC-Fu^{EE}* exhibited higher levels of Ci^F staining than wing discs expressing *MS1096>CC-Fu* (arrows in Fig. 2.5B-D, column 2). A likely explanation is that Fu^{EE} and CC-Fu^{EE} but not CC-Fu could effectively block Ci processing. To test this possibility, we applied an *in vivo* assay for Ci processing. When *UAS-Ci* was misexpressed in wing discs that carry *smo* mutant clones and a *Hh-lacZ* reporter gene, the expression of *Hh-lacZ* in P-compartment *smo* mutant cells was blocked due to Ci being processed into Ci^R in these cells (Jia et al. 2005, Methot and Basler 1999). Coexpression of Fu or CC-Fu did not significantly alleviate the blockage of *Hh-lacZ* expression in *smo* mutant cells (arrows in Fig. 2.5E-F"), indicating that neither Fu nor CC-Fu was able to efficiently block Ci processing. In contrast, coexpression of Fu^{EE} partially whereas CC-Fu^{EE} more completely derepressed the *Hh-lacZ* expression in posterior *smo* mutant cells (arrows in Fig. 2.5G-H"). Of note, Fu^{EE} or CC-Fu^{EE} derepressed *Hh-lacZ* expression less efficiently in ventrally situated *smo* mutant clones (arrowheads in Fig. 2.5G-H"), likely due to lower levels of transgene expression in this region. In keeping with this dosage effect, increasing the expression level of CC-Fu by growing larvae at 30 ^{0}C rendered partial inhibition of Ci processing (data not shown). Taken together, these results demonstrate that active forms of Fu inhibit Ci^R production with Fu^{EE} and CC-Fu^{EE} being more effective than CC-Fu.

Activated Fu attenuates Ci-Sufu complex formation

To determine whether activated Fu converts Ci^F to Ci^A through attenuating the formation of Ci-Sufu complex that normally inhibits Ci activity (Methot and Basler 2000, Ohlmeyer and Kalderon 1998, Wang G. et al. 2000b), we examined the effect of different forms of Fu on the interaction between Sufu and Ci^{-PKA}, a Ci variant with three PKA sites (S838, S856 and S892) mutated to Ala and thus no longer processed but still inhibited by Sufu (Smelkinson et al. 2007, Wang G. et al. 1999). Using immunoprecipitation assay, we found that HA-CC-Fu, HA-CC-Fu^{EE} and HA-Fu^{EE} markedly decreased the amount of Flag-Sufu pulled down by Myc-Ci^{-PKA} (Fig. 2.6C, compare lanes 4, 5 and 7 with lane 3)

whereas neither HA-CC-Fu^{G13V} nor HA-Fu altered the association between Myc-Ci^{-PKA} and Flag-Sufu (Fig. 2.6C, lanes 6 and 8). Consistent with this reduced interaction between Ci^{-PKA} and Sufu, Dr. Shuang Li observed that active forms of Fu demolished the cytoplasmic co-localization between N-terminally CFP-tagged Ci^{-PKA} (CFP-Ci^{-PKA}) and C-terminally YFP-tagged Sufu (Sufu-YFP) as well as dramatically decreased the FRET between them (Fig. 2.6D,E).



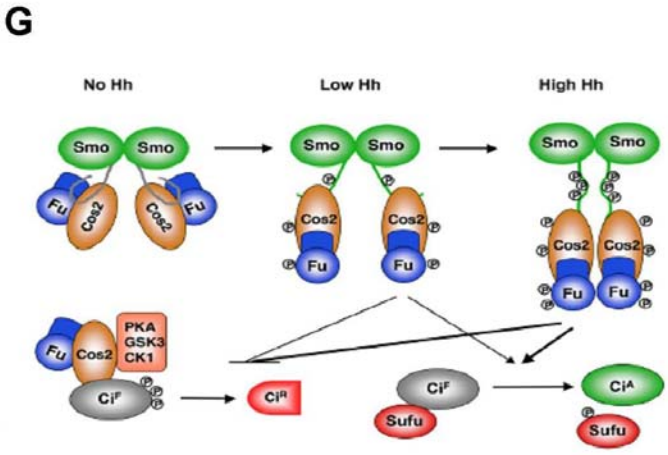


Figure 2.6 Active forms of Fu dissociate Ci from Sufu (Results in D, E were generated by Dr. Shuang Li). (A) S2 cells were transfected with Flag-Sufu and different HA-tagged Fu variants. Cells extracts without or with λ-pp treatment were run on a 12.5% (120:1 acrylamide:Bis-acrylamide) Anderson gel at 140V followed by western blot with an anti-Flag antibody (Top panel), and run on a 6% (29:1 acrylamide:Bis-acrylamide) gel at 90 V followed by western blot with an anti-HA antibody (Bottom panel). Active forms of Fu but not CC-Fu^{G13V} or Fu induced a mobility shift of Flag-Sufu. (B) S2 cells were transfected with Myc-Ci^{-PKA} and different HA-tagged Fu variants. Cells extracts without or with λ-pp treatment were run on 6% (29:1 acrylamide:Bis-acrylamide) gels at 90 V followed by western blot with an anti-Myc antibody (Top panel) and an anti-HA antibody (Bottom panel). Active forms of Fu but not CC-Fu^{G13V} or Fu induced a mobility shift of Myc-Ci^{-PKA}. (C) Western blots of immunoprecipitates (Top two panel) and lysates (Bottom two panels) from S2 cells transfected with indicated constructs and treated with 50uM MG132 for 4 hours before harvesting. The asterisk indicates IgG. (D) S2 cells were transfected with CFP-Ci^{-PKA} and Sufu-YFP without or with indicated Fu variants. Transfected cells were treated with LMB before harvesting. (E) FRET efficiency between CFP-Ci^{-PKA} and Sufu-YFP expressed in S2 cells in the absence or presence of indicated Fu variants. (F) A model for Fu activation through Hh-induced Smo conformational switch. In the absence of Hh, Cos2/Fu forms an inactive complex with Smo to inhibit Smo phosphorylation. Cos2 recruits multiple kinases to phosphorylate Ci^{F}, leading to Ci processing to Ci^{R}. Hh signaling triggers Smo phosphorylation, cell surface accumulation and conformational change, leading to the assembly of active Smo-Cos2-Fu complexes. Hh-induced clustering of Smo C-tails promotes Fu kinase domain dimerization, phosphorylation and activation. Graded Hh signals progressively increase Fu dimerization and phosphorylation, leading to a gradual increase of Fu activity. Activated Fu inhibits Ci processing to Ci^{R} and coverts Ci^{F} to Ci^{A} by regulating Ci-Cos2-kinase and Ci-Sufu complex formation. Low levels of Hh may initiate Fu activation by a dimerization independent mechanism.

A likely mechanism for activated Fu triggering the dissociation of Sufu from Ci could be to modulate the interaction surface by phosphorylating Sufu, Ci or both. In line with a previous finding that Fu is required for Hh-induced Sufu phosphorylation (Lum et al. 2003b), we found that HA-CC-Fu, HA-CC-Fu^{EE} or HA-Fu^{EE} but not HA-CC-Fu^{G13V} or HA-Fu induced a mobility shift of Flag-Sufu that was abolished by phosphatase treatment (Fig. 2.6A). Remarkably, we also found that active forms of Fu but

not HA-CCFu^{G13V} or HA-Fu induced a mobility shift of Myc-Ci^{-PKA} that was abolished by phosphatase treatment (Fig. 2.6B), suggesting that Fu induces phosphorylation of Ci at sites distinguished from previously identified PKA/CK1 sites that are involved in negative regulation of Ci activity (Smelkinson et al. 2007, Wang G. et al. 1999). Thus, activated Fu promotes phosphorylation of both Sufu and Ci, which may contribute to the dissociation of Sufu from Ci as well as the conversion of Ci^{F} to Ci^{A}.

Discussion

A long-term puzzle in the field is how the membrane-associated GPCR like protein Smo relays the Hh signal to the downstream transcription factor, Ci. Recent Study by David Robbins' group revealed that Smo can function as a canonical GPCR, signaling through Gαi to regulate Hh pathway activation (Ogden et al. 2008); while in this study we demonstrate mechanisms underlying a GPCR-independent cascade, in which Smo activates Ci through the cytoplasmic Fu/Cos2 signaling complex. It is known that Smo binds to Cos2/Fu in both quiescent cells and Hh-stimulated cells, and the interaction is mediated by two distinct regions in Smo C-tail: a membrane proximal domain and a C-terminal region (Jia et al. 2003, Lum et al. 2003b). The finding that deleting the membrane proximal domain potentiated (Jia et al. 2003) whereas deleting the C-terminal region impaired Smo activity (Claret et al. 2007, Jia et al. 2009, Liu Y. et al. 2007) raises an interesting possibility that Smo/Cos2/Fu may interact in a dynamic manner and distinct complexes with differential activities may exist depending on the Hh signaling status. In support of this model, previous study by Jia's group demonstrated that, in the absence of Hh, the membrane proximal domain recruits Cos2/Fu/PP4 to inhibit Smo activity via interfering its phosphorylation and cell surface accumulation (Jia et al. 2009); while here we show that, upon Hh stimulation, Cos2 recruits Fu to the C-terminal region of Smo C-tail, which activates pathway via triggering dimerization and activation of Fu kinase by the conformational switch and clustering of Smo C-tails (Fig. 2.6G).

In keeping with previous observations that clustering of Smo C-tails or Hh stimulation results in Fu phosphorylation (Lum et al. 2003b, Therond P. P. et al. 1996b, Zhao et al. 2007a), we found that

dimerization-mediated activation couples with Fu self-phosphorylation likely due to auto-phosphorylation since it depends on Fu kinase activity and an active form of Fu can *tran*-phospohrylate a kinase-dead form of Fu. Furthermore, by mutagenesis and developing phospho-antibodies, we found that, like many other kinases (Nolen et al. 2004, Pike et al. 2008), Fu phosphorylation occurs, at least partially, at its AL. Intriguingly, we also found that gradually increasing Smo phosphorylation or Hh concentration progressively increases dimerization, AL phosphorylation, as well as mobility shift of Fu kinase (data not shown), implying that more species of Fu or more sites in one Fu molecule could get phosphorylated upon increased signal strength. Previously, there are cases that increasing phosphorylation in one kinase protein correlates with a stepwise increase in the kinase activity (Chong et al. 2001, Favelyukis et al. 2001). Thus, Hh gradient may elevate Fu kinase activity in a dose dependent manner, likely as a consequence of phosphorylation-induced gradual conformational change and dimerization of Smo C-tails (Fig. 2.6G). In line with this notion, a recent study using the phospho-specific antibody against Cos2 Ser572, the only identified phosphorylation site for Fu, revealed a grade of Fu kinase activity that corresponds to the Hh gradient along the A/P boundary of *Drosophila* wing discs (Raisin et al. 2010).

The observation that Fu kinase activity could be induced even by low levels of Hh raised an interesting possibility that Fu may contribute to all levels of Hh signaling in contrast to the conventional view that Fu is only required for high levels of Hh signaling. Although *fu* mutations only affect high but not low threshold Hh responsive genes (Alves et al. 1998), it could be explained by that none of *fu* mutations examined so far represents a null mutation or that paralleled mechanisms, such as Gαi (Ogden et al. 2008), could mask the contribution of Fu to low levels of Hh signaling. To examine the role of Fu in transducing low levels of Hh signaling, we performed an *in vivo* assay for Ci processing as low levels of Hh signaling suffice to block Ci processing. Indeed, we found that active forms of Fu can block Ci processing into Ci^{R}, and thus provide the first evidence that Fu involves in tranducing low levels of Hh signaling.

Although the essential role of Fu in tranducing high levels of Hh signaling is well-known, the underlying biochemical mechanism remains elusive. Here consistent with previous genetic studies that showed Fu kinase is required for the conversion of Ci^F to Ci^A by antagonizing Sufu (Ohlmeyer and Kalderon 1998), we observed that active Fu converts Ci into a hyperactive but liable form that can be antagonized by coexpressing Sufu in *Drosophila* wing discs; and furthermore we demonstrated that active Fu dissociates Ci from Sufu, probably by phosphorylating both Ci and Sufu. In keeping with our findings, recent studies using mammalian cultured cells revealed that Shh signaling induces dissociation of full-length Gli proteins from Sufu (Humke et al. 2010, Tukachinsky et al. 2010), suggesting that inhibition of Sufu-Ci/Gli complex formation could be a conserved mechanism mediating the conversion of Ci^F to Ci^A.

While our manuscript was under review, Daniel Kalderon's group published a paper where they made independent observations that Hh activates Fu through phosphorylation to elicit a full spectrum of pathway responses (Zhou and Kalderon 2011).

Material and Methods

Mutations and transgenes

smo^3 and *$cos2^2$* are null or strong alleles and have been described (Chen Y. and Struhl 1998, Grau and Simpson 1987). *UAS-Ci^U*, *UAS-Ci*, *UAS-Sufu*, *dpp-lacZ* and *Hh-lacZ* have been described (Methot and Basler 1999, Wang G. et al. 2000b). *MS1096*, *C765 and ap-Gal4* drivers have been described (Chen Y. et al. 2010, Wang G. et al. 1999). The *vas-phi-zh2A-VK5* flies were used to generate transformants inserted at the 75B1 *attP* locus (Bischof et al. 2007). Smo-CFP and Cos2-YFP contain CFP or YFP fused in-frame to their C-termini. CFP-Fu and YFP-Fu contain CFP or YFP fused in-frame to the N-terminus of Fu. Both CFP-Fu and YFP-Fu can rescue *fu* mutant phenotypes in adult wings (data not shown). Smo SA, SD and RA mutants have been described (Jia et al. 2004, Zhao et al. 2007a). CFP-Ci contains CFP fused in-frame to the N-terminus of Ci. Sufu-YFP contains YFP fused in-frame to the C-terminus of Sufu. All CFP/YFP fusion constructs were generated by subcloning the corresponding coding sequences into

pUAST vectors. To generate CC-Fu and CCm-Fu, peptides corresponding to the wild-type or mutant leucine zipper of the yeast GCN4 (Fig. 2.2A) followed by a flexible linker (GSSG) were generated by multi-step PCR and subcloned between BglII and NotI sites of *pUAST-Myc* and *pUAST-HA* (Tong and Jiang 2007). For generating transformants, the coding regions for HA-Fu, HA-Fu^{EE}, HA-CC-Fu, HA-CC-Fu^{EE} and HA-CCm-Fu were subcloned between EcoRI and XbaI sites of a modified *pUAST* vector with an attB sequence inserted upstream of the *UAS*-binding sites (Liu Y. et al. 2007).

Generation of mutant clones

FRT/FLP-mediated mitotic recombination was used to generate mutant clones as previously described (Jiang and Struhl 1995). The genotypes for generating clones are as follows. *smo^3* mutant clones expressing *UAS-Ci^U* and *UAS-CC-Fu* transgenes: *MS1096 hsp-flp1/yw or Y; smo^3 FRT40/FRT40 ,hsp-GFP; UAS-Ci^U, UAS-CC-Fu/+*. *smo^3* mutant clones expressing *UAS-Ci* and Fu transgenes: *MS1096 hsp-flp1/yw or Y*; *smo^3 FRT40/FRT40, hsp-GFP*; *UAS-HA-Ci, (UAS-Fu, UAS-CC-Fu, UAS-Fu^{EE}, UAS-CC-Fu^{EE}) /Hh-lacZ*.

Imaginal disc staining and LMB treatment

Standard protocols for immunostaining of imaginal discs were used (Jiang and Struhl 1995). Antibodies or dyes used for this study are rat anti-Ci antibody, 2A1 (Motzny and Holmgren 1995), mouse anti-Ptc antibody (DSHB), mouse anti-En antibody (DSHB), mouse anti-HA antibody (Santa Cruz), rabbit anti-LacZ antibody (Affinity Bioreagents), mouse anti-LacZ antibody (Sigma), rabbit anti-GFP antibody (Invitrogen) and DRAQ5 (Cell signaling). In experiments involving LMB treatment, late third instar larvae were dissected and cultured in cl-8 cell medium containing 100nM LMB for 4 hr before fixation.

Cell culture, transfection, immunostaining, immunoprecipitation and western blot analysis

Drosophila S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100mg/ml of streptomycin at 24^{o}C. Transfection was carried out by Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. Hh-conditioned medium treatment was carried out as described (Lum et al. 2003a). Briefly, HhN stable expression S2 cells were selected in 200 g/ml hygromycine (Roche). Hh-conditioned medium was prepared by culturing cells without hygromycine and induced with 0.7 mM $CuSO_4$ for one day. The medium was harvested and sterilely filtered. Unless mentioned otherwise, Hh-conditioned medium was used at a 6:4 dilution ratio by fresh medium (referred to as 100% Hh in Fig. 2.4I). Immunoprecipitation and western blot analyses were carried out using standard protocols as previously described (Zhang W. et al. 2005). For immunostaining, S2 cells were harvested and washed with PBS, fixed with 4% formaldehyde at room temperature for 20 minutes, and incubated with the first antibody for 2 hours. Then, cells were washed three times with PBS followed by the secondary antibody staining for 1.5 hours. Images were captured by confocal microscopy and signals were quantified by ImageJ software. Phospho-Fu antibodies were generated by Genemed Synthesis (San Antonio, TX) with the following phospho-peptides as antigens: CDFGLARNMT(p)LGT(p)HVL (for p-T151/T154) and HVLT(p)S(p)IKGTPLYMAPE (for p-T158/S159). Phospho-antibodies were purified by positive and negative selections using the phospho-peptides and nonphospho-peptides affinity columns.

Luciferase assays

For *ptc-luc* reporter assays, S2 cells were transfected with 1ug *ptc-luc* reporter construct and 50ng RL-PolIII renilla construct in 12 well plates together with 0.5ug Ci, 0.25ug Sufu, and 0.5ug Fu constructs. After 48 hours incubation, the reporter assays were performed using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed in triplicate using FLUOstar OPTIMA (BMG LABTECH).

RNAi in *Drosophila* S2 cells

Double-stranded RNA (dsRNA) was generated by MEGAscript High Yield Transcription Kit (Ambion) according to the manufacturer's instruction. dsRNA targeting Cos2 was generated according to Lum et al (Lum et al. 2003a). dsRNA targeting the Firefly Luciferase coding sequence was used as a control. For RNAi knockdown experiments, S2 cells were cultured in serum-free medium containing the indicated dsRNA for 8 h at 24°C. After adding fetal bovine serum to a final concentration of 10%, dsRNA-treated cells were cultured for 24 hours before transfection. 48 hours after transfection, the cells were collected for analysis.

FRET analysis

FRET analysis was carried out as previously described (Zhao et al. 2007a). CFP/YFP-tagged constructs were transfected into S2 cells together with an *ub-Gal4* expression vector. Cells were washed with PBS, fixed with 4% formaldehyde for 20 minutes and mounted on the slides in 80% glycerol. CFP signals were acquired with 100X objective of Zeiss LSM510 confocal microscope before (BP) and after (AP) photobleaching YFP. Each data set was calculated using 10-20 individual cells. In each cell, four or five regions of interest in photobleached area were selected for analysis. The intensities of CFP signals were quantified by ImageJ software. The FRET efficiency was calculated using the formula: $FRET\% = [(CFP_{AP} - CFP_{BP})/CFP_{AP}] \times 100$. Of note, only CFP signals that colocalized with YFP signals (both membrane and intracellular) were selected for calculation. For FRET analysis in wing discs, CFP/YFP-tagged *UAS* transgenes were expressed using *MS1096* Gal4 driver. CFP signals were acquired with 63X objective of Zeiss LSM510 confocal microscope before and after photobleaching YFP.

CHAPTER III

Characterization of the transcriptional repression domain in Ci

Introduction

Transciptional repressors play important roles in vital biological processes (Affolter et al. 2008). Usually, a functional transcriptional repressor contains a DNA-binding domain that interacts with specific promoter elements and small portable repression domain(s) that represses gene transcription (Hanna-Rose and Hansen 1996). Recent studies have elucidated a variety of mechanisms for transcriptional repressors to achieve active repression, which include interference with the formation or activity of the basal transcription complex, competition with activators or coactivators for access to DNA, quenching the transcriptional stimulation of an activator bound to the same promoter, and recruitment of corepressor proteins such as chromatin remodeling machine (Cowell 1994, Hanna-Rose and Hansen 1996, Johnson 1995). Notably, some repressors can employ more than one operating mechanism depending on the promoter context (Hanna-Rose and Hansen 1996).

Ci, the transcription factor of *Drosophila* Hh pathway, is phosphorylated by Cos2-recruited multiple kinases in the absence of Hh, and targeted to the Slimb-Cul1-based E3 ligase complex for ubiquitin/proteasome-mediated proteolysis to generate a truncated form, Ci^R. (Jia et al. 2002, Jia et al. 2005, Price and Kalderon 2002, Smelkinson et al. 2007, Zhang W. et al. 2005). Ci^R, lacking the transactivation domain but retaining the DNA-binding domain, enters nucleus and functions as a transcriptional repressor to turn off the expression of Hh target gene, such as *dpp* (Aza-Blanc et al. 1997, Methot and Basler 1999). The molecular mechanism underlying the Ci^R-mediated transcriptional repression is not clear, and whether Ci^R contains a functional repression domain remains to be determined.

In this study we found that N-terminus (aa 1-440) of Ci has transcriptional repression activity. It attenuates the transcriptional activity of fused Gal4 activation (GA) domain and represses *hh-LacZ* when expressed in *Drosophila* wing discs. By making a series of truncations, we demonstrated that the N-terminus of Ci contains multiple repression elements, which can function independently and exhibit differential repression activity in different regions of wing discs. Interestingly, we observed that Sufu, a well-known inhibitor thought to bring a corepressor(s) to Ci/Gli proteins (Cheng S. Y. and Bishop 2002), is dispensable for the repression activity of Ci N-terminus. Thus, it would be interesting to study the Sufu-independent mechanism(s) that may be employed by the N-terminal repression domain of Ci in future.

Results

The N-terminus of Ci has transcriptional repression activity

To study how Ci activity is regulated, we generated Ci-GA chimeric proteins by fusing different Ci fragments to GA domain. CiZnGA contains Zn finger DNA-binding domain of Ci whereas CiGA contains an additional N-terminal region (aa 1-440) of Ci (Fig. 3.1A). Expressing CiZnGA or CiGA in cl-8 cells activated a *ptc-luc* reporter to a level comparable to expressing wild-type Ci or Hh stimulation (Fig. 3.1A); however, when expressed in *Drosophila* wing discs using *MS1096* Gal4 driver, CiZnGA ectopically activated *ptc* in both A- and P-compartment cells whereas CiGA didn't induce any *ptc* expression in P-compartment cells even there is high levels of Hh (Fig. 3.1B), implying that the Ci N-terminal region included in CiGA but not CiZnGA has an ability to compromise the activity of GA domain when expressed in *Drosophila* wing discs. Furthermore, when expressed in clones situated in P-compartment cells using *actin* Gal4 driver, CiGA but not CiZnGA behaved similarly as Ci^R to repress the expression of Hh-LacZ (Fig. 3.1C; data not shown) (Jia et al. 2002, Methot and Basler 1999). Taken together, we concluded that the N- terminus of Ci has transcriptional repression activity.

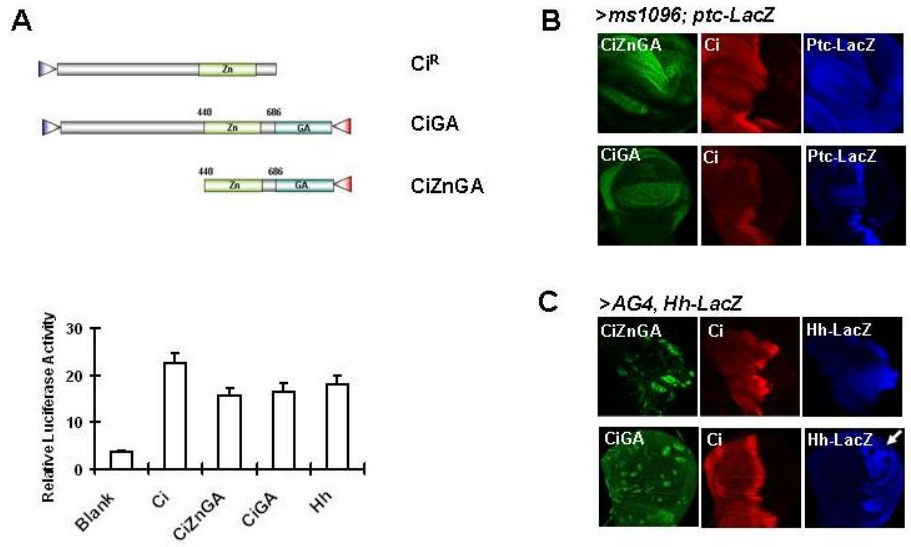


Figure 3.1 The N terminus of Ci has transcriptional repression activity. (A) Up: Diagrams of Ci^R, CiGA and CiZnGA constructs. Green boxes represent Zn finger DNA-binding domain. Blue boxes represent Gal4 transactivation domain. Blue triangles represent HA tag. Red triangles represent Myc tag. Bottom: a *ptc-luc* reporter assay in cl-8 cells transfected with indicated Ci constructs or treated with Hh conditional medium. The *y* axis represents normalized *ptc-luc* activity. Wild-type Ci, CiZnGA, and CiGA activated *ptc-luc* reporter in cl-8 cells to a level comparable with Hh stimulation. (B) Wing discs expressing *UAS-CiZnGA-Myc* or *UAS-HA-CiGA-Myc* with *MS1096* Gal4 driver at 25^0C were immunostained to show the expression of Myc (green), Ci (red) and Ptc-LacZ (blue). Expressing CiZnGA induced ectopic expression of Ptc-LacZ in both A- and P-compartment cells, whereas expressing CiGA only induced slight activation of Ptc-Lac in A-compartment cells. (C) Wing discs with clones expressing *UAS-CiZnGA-Myc* or *UAS-HA-CiGA-Myc* with *actin-Gal4 (AG4)* driver at 25^0C were immunostained to show the expression of Myc (green), Ci (red) and Hh-LacZ (blue). An arrow indicates the expression of Hh-LacZ was repressed in clones expressing CiGA.

Sufu is dispensable for the repression activity of Ci N-terminus

Sufu is one of the well-known inhibitors of Ci. It impedes Ci nuclear import by forming a cytoplasmic complex with Ci as well as blocks the conversion of Ci^F to Ci^A likely through entering the nucleus together with Ci and recruiting a corepressor (s) (Cheng S. Y. and Bishop 2002, Methot and Basler 2000, Monnier et al. 1998, Ohlmeyer and Kalderon 1998, Wang G. et al. 2000b). Previous study

has shown that Sufu binds to a "SYGH" motif located in the N-terminal highly conserved domain of Ci/Gli proteins (Fig. 3.2A) (Huntzicker et al. 2006), which raises an interesting possibility that the repression activity of Ci N-terminus is achieved by recruiting a corepressor (s) through Sufu.

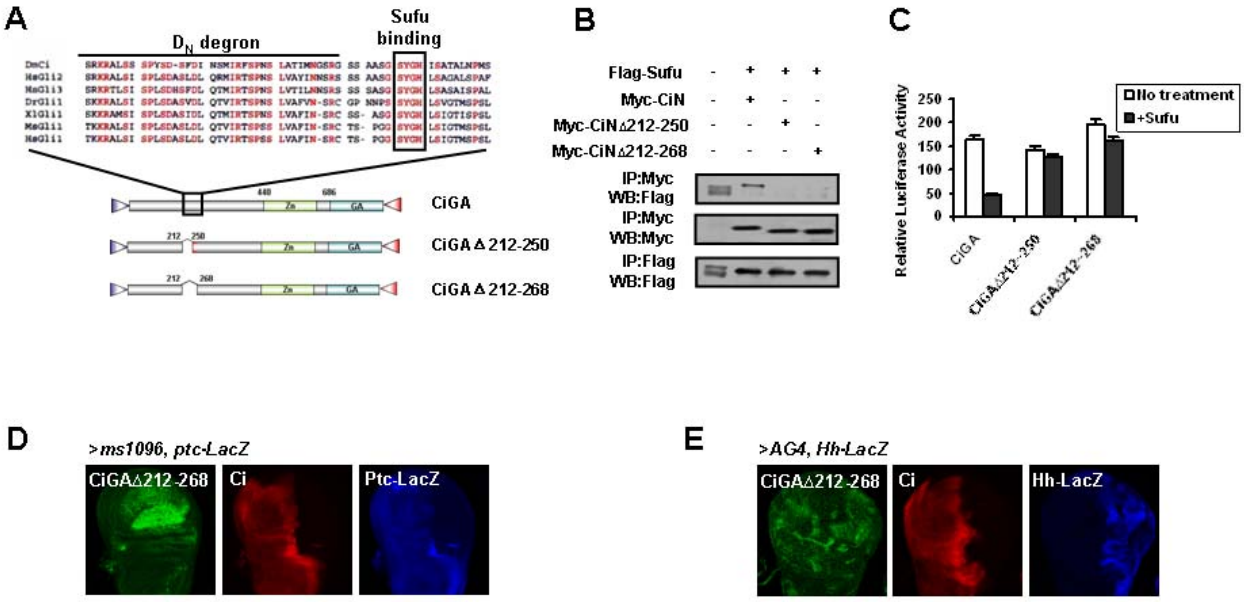


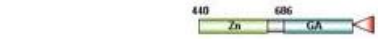
Figure 3.2 Sufu is dispensable for the function of N-terminal repression domain. (A) Diagrams of CiGA and its internal deletion variants with sequence alignment of the N-terminal conserved domain of Ci/Gli proteins shown above. CiGA Δ 212-250 deletes the D_N degron but retains the Sufu binding motif, whereas CiGA Δ 212-268 deletes the entire N-terminal conserved domain. Green boxes represent Zn finger DNA-binding domain. Blue boxes represent Gal4 transactivation domain. Blue triangles represent HA tag. Red triangles represent Myc tag. (B) Cell lysates from S2 cells transfected with indicated constructs were subjected to imunoprecipitation followed by Western blot with indicated antibodies. (C) A *ptc-Luc* reporter assay in S2 cells transfected with indicated CiGA constructs in the absence or presence of Flag-Sufu. The *y* axis represents normalized *ptc-luc* activity. (D) A wing disc expressing *UAS-HA-CiGA Δ 212-268-Myc* with *MS1096* Gal4 driver at 25^0C was immunostained to show the expression of HA (green), Ci (red) and Ptc-LacZ (blue). Expressing CiGA Δ 212-268 did not induce ectopic activation of *ptc-LacZ*. (E) A wing disc with clones expressing *UAS-HA-CiGA Δ 212-268-Myc* with *actin-Gal4* driver at 25^0C was immunostained to show the expression of HA (green), Ci (red) and Hh-LacZ (blue). The expressing of Hh-LacZ was still repressed in clones expressing CiGA Δ 212-268.

To test this possibility, we generated internal deletion variants in both CiGA and Myc-CiN background, with Δ212-268 deleted the entire N-terminal conserved domain including both D_N degron and Sufu-binding motif and with Δ212-250 retaining the Sufu-binding motif (Huntzicker et al. 2006) (Fig. 3.2A, B). By performing immunoprecipitation analysis, we found that Sufu interacts with CiN but not CiN Δ212-268, which is in line with previous observation that N-terminal conserved domain of Ci is essential for Sufu binding (Fig. 3.2B). Of note, CiN Δ212-250, although retaining the Sufu-binding motif, failed to pull down Sufu (Fig. 3.2B), suggesting that the intactness of adjacent region is also important for Sufu binding. Consistent with this, coexpressing Sufu repressed the activity of CiGA but not CiGA Δ212-268 or CiGA Δ212-250 in a *ptc-luc* reporter assay (Fig. 3.2C). Thus, we created two variants of CiGA that do not interact with Sufu. However, when expressing CiGA Δ212-268 in *Drosophila* wing discs, it still behaved like a transcriptional repressor as it failed to induce ectopic activation of *ptc* as well as repressed Hh-LacZ expression (Fig. 3.2D, E), indicating that the N-terminal conserved domain and Sufu are dispensable for the repression activity of Ci N-terminus.

Multiple repression elements exist in the N-terminus of Ci

To map the miminal repression element(s) outside the N-terminal conserved domain, we made a series of truncated CiGA variants (Table 3.1) and examined their ability to activate *ptc* and repress *Hh-LacZ* in *Drosophila* wing discs. First, by dividing the N-terminal non-conserved region into two big fragments, we found that CiGA Δ212-440 has similar repression activity as CiGA whereas CiGA Δ1-268 exhibits dramatic reduction of repression activity as it induced ectopic expression of *ptc* in A- and P-compartment cells and did not repress *Hh-LacZ* expression in the wing pouch region (Table 3.1). In addition, consistent with the observation that the N-terminal conserved domain is dispensable for the repression activity of N-terminus of Ci, CiGA Δ1-212 including the N-terminal conserved domain didn't show any difference of repression activity compared with CiGA Δ1-268 (Table 3.1). We then made

further truncations of aa 1-212 given this region having strong repression activity, and found that Ci1-75ZnGA, Ci1-150ZnGA and Ci104-212ZnGA are able to eptically activate *ptc* in A- and P-compartment cells albeit they still efficiently inhibit *Hh-LacZ* expression (Table 3.1). Therefore, the N terminus of Ci appears to contain multiple independent elements with differential repression activity.

	Ms1096 ptcAT/cyo		AG4 ptcAT/cyo		AG4 HhLacZ/TM6
	A	P	A	P	
 CiZnGA	Y	Y	Y	Y	N(not repress)
 CiGA	Y	N	Y	N	R(repress)
 Ci^{R}			N	N	R
 CiGAΔ212-250	N	N			R
 CiGAΔ212-268	N	N	N	N	R
 CiGAΔ212-440	Y	N	Y	N	R
 CiGAΔ1-268	Y	Y	Y	Y	N in wing pouch; R around it
 CiGAΔ1-212	Y	Y	Y	Y	N in wing pouch; R around it
 Ci104-212ZnGA	Y	Y	Y	Y	R
 Ci1-75ZnGA	Y	Y	Y	Y	R
 Ci1-150ZnGA	Y	Y	Y	Y	R
 Ci828CBP	Y	Y	Y*	Y*	

* yw122 PtcAT/cyo(y+); 745.5/TM6B female x Ci828CBP male

Table 3.1 Mapping repression element(s) in the N terminus of Ci. Column 1: Diagrams of Ci^R, CiGA, CiGA truncation variants, and Ci828CBP constructs. Green boxes represent Zn finger DNA-binding domain. Blue boxes represent Gal4 transactivation domain. Red boxes represent the dCBP interacting domain, which is the transactivation domain of Ci. Blue triangles represent HA tag. Red triangles represent Myc tag. Columns 2-6: A summary of the *in vivo* behaviors of corresponding constructs. Columns 2 and 3 indicate the ability of indicated Ci variants to activate *ptc* expression in A- (column 2) or P- (column 3) compartment cells when expressed by *MS1096* Gal4 driver at 25^0C. Y represents "ectopic activation of *ptc* expression". N represents "no ectopic activation of *ptc* expression". Columns 4 and 5 indicate the ability of indicated Ci variants to activate *ptc* expression when expressed by *actin-Gal4* driver in A- (column 4) or P- (column 5) compartment clones at 25^0C. Y represents "ectopic activation of *ptc* expression". N represents "no ectopic activation of *ptc* expression". Column 6 indicates the ability of indicated Ci variants to repress *Hh-LacZ* expression when expressed by *actin-Gal4* driver in P-compartment clones at 25^0C. R represents "repression on *Hh-LacZ* expression". N represents "no repression on *Hh-LacZ* expression".

Discussion

In this study, we demonstrated that the N-terminal aa 1-440 of Ci can function as a potential transcriptional repression domain. We provided evidence that the strongest repression activity is associated with aa 1-212 of Ci, which is in line with a recent finding that the N-terminal part of Gli3, one of the mammal homologs of Ci, harbors gene repression activity with the minimal repression domain mapped to aa 106-236 (Tsanev et al. 2009). Moreover, we observed that aa 1-212 can be further divided into smaller fragments with the ability to inhibit *Hh-LacZ* expression independently and that aa 268-440, although not repressing *Hh-LacZ* in the wing pouch region, still efficiently represses *Hh-LacZ* outside the wing pouch, implying that multiple repression elements exist in Ci N-terminus and they harbor differential repression activity depending on the position where they express in wing discs.

Previously, people have identified a myriad of transferable transcriptional repression domains, which, according to the primary amino acid content, can be loosely categorized into Ala-rich, Pro-rich,

Ala- and Pro-rich, charged, hydrophobic, or unique repression motifs (Hanna-Rose and Hansen 1996). In line with this, we found that two Ala clusters (aa 25-32, aa 172-179) locate in the N-terminal region (aa 1-212) of Ci that harbors the strongest repression activity, and that the corresponding regions in Gli proteins, although not containing the Ala clusters, are highly rich in Pro residues. So far, the function of Ala clusters in mediating transcriptional repression remains uncertain. In the first-indentified Ala-rich repression domain in the product of the *Drosophila* gene *Krüppel*, the Ala motif is not required for its repression function but instead serves a structural role to nucleate an α-helix that contains a Glu residue critical for its repression activity (Licht et al. 1994); whereas in the cases of *engrailed* and *Knirps*, Ala motifs are essential for their repression activity (Gerwin et al. 1994, Han K. and Manley 1993). Here, we found that internally deleting the two Ala clusters did not significantly affect the repression activity of CiGA Δ 212-440 (data not shown), indicating that they are not required for the function of N-terminal repression domain of Ci.

Then the question is how the N-terminus of Ci exerts the repression activity. Interestingly, by C-terminally fusing the CBP binding domain (aa 1020-1160) of Ci that is known to recruit CBP/P300 histone acetyltransferase (HAT) complex (Akimaru et al. 1997) to Ci N-terminus, we compromised its repression function likely through the HAT activity (Table 3.1). In addition, recent studies revealed that Sufu, a negative regulator binding to the N-terminal conserved domain of Ci/Gli proteins, associates with SAP18, a component of mSin3-histone deacetylase (HDAC) corepressor complex (Cheng S. Y. and Bishop 2002). All these observations raise an interesting possibility that the Ci N-terminal repression domain may recruit HDAC corepressor proteins through Sufu-SAP18. Nevertheless, after generating a CiGA variant with the N-terminal conserved domain deleted and thus neither binding to Sufu nor responding to Sufu-mediated repression, we found that both the N-terminal conserved domain and Sufu are dispensable for the repression activity of Ci N-terminus. One explanation could be that Sufu is not required for the interaction between corepressor(s) and Ci N-terminus. Indeed, by yeast two-hybrid and

immunoprecipitation assays, we found that *Drosophila* SAP18 can bind to Ci N-terminus directly (data not shown). Another favorable explanation is that other part of Ci N-terminus, which has been shown containing multiple repression elements, may recruit redundant corepressor(s) such that abrogating Sufu-dependent corepressor(s) only is not sufficient to achieve derepression. Lastly, it remains possible that the N-terminus of Ci mediates transcriptional repression by employing mechanisms other than or in addition to recruiting corepressor(s), such as interference with the basal transcription machinery or competition with transcriptional activators.

The possibility of redundancy makes it difficult to indentify corepressor(s) through genetic loss of function study. Indeed, in a candidate gene screen, we failed to observe any derepression by simply knocking down single HDAC or its interacting partner when CiGA was coexpressed (Table 3.2). Another disadvantage for loss of function approach is that some candidate genes may play dual roles in Hh pathway such that the derepression caused by dysfunction of a gene could be masked by the loss of its positive effects in the pathway. For example, we found that HDAC1 RNAi and its binding partner Sin3A RNAi reduced the pathway activity instead of leading to derepression (Table 3.2), which could be explained by a recent finding that HDAC1 complex functions as a positive regulator of Hh pathway by mediating deacetylation of Gli1/2 and promoting their transcriptional activity (Canettieri et al. 2010). Given the above complexity, in future it would be favorable to identify candidates more specifically from proteins binding to the N-terminus of Ci using biochemical purification followed by mass spectrometry. Then, we can knock down candidates in combination in the background of CiGA coexpression, in a hope to see derepression by avoiding the redundancy of *trans*-corepressor factors. Alternatively, we can map the minimal binding motif of a candidate in Ci N-terminus, and examine whether knocking down individual candidate could result in derepression of a CiGA truncation variant that harbors the corresponding binding motif of that candidate by limiting the redundancy of *cis*-repression elements.

Candidate Genes	***MS1096; Dicer2; CiGA/TM2***
VDRC30600: HDAC1	Abolish ectopic *ptc* expression in A-compartment cells and attenuate *ptc* expression at A/P boundary
VDRC 46930: HDAC1	
VDRC 20814: HDAC3	No effect
VDRC 20522: HDAC4	No effect
VDRC 13687: Gug	No effect
VDRC 23201: Sir2	No effect
VDRC 21999: Sirt2	No effect
VDRC 10808: Sin3A	Similar to HDAC1
NIG6046: dSap18	No effect

Table 3.2 A candidate gene screen for corepressor(s) of Ci. Column 1 lists the RNAi stock ID and the name of candidate genes. VDRC: Vienna Drosophila RNAi Center; NIG: National institute of Genetics of Japan. Column 2 lists the effect of candidate genes on the activity of CiGA. When coexpressed with CiGA using *MS1096* Gal4 driver at 25^0C in the presence of Dicer2, a factor promoting RNAi efficiency, HDAC1 RNAi and Sin3A RNAi abolished the CiGA-induced ectopic *ptc* expression in A-compartment cells and attenuated *ptc* expression along the A/P boundary; whereas other candidate gene RNAi had no obvious effect on *ptc* expression.

Finally, the biological roles of the N-terminal repression domain in regulating Ci activity remain to be determined. It is possible that the N-terminal repression domain is essential for the truncated Ci^R to function as a transcriptional repressor. It is also possible that the N-terminal repression domain could compromise the activity of Ci^F and lock it in a default status in the absence of high levels of Hh. Hence, further efforts to clarify the mechanisms underlying the repression function of Ci N-terminus and also how the repression activity is regulated by Hh signaling will have important implications in our understanding how Ci^R works as well as how the conversion of Ci^F into hyperactive Ci^A is achieved. Moreover, given the conservation of N-terminal repression domain in Gli3 (Tsanev et al. 2009), our findings will also expand the knowledge on the regulation of Gli proteins.

Material and Methods

Mutations and transgenes

UAS-CiZnGA-Myc, UAS-Ci^{R}, *hsp-flp1*, *ptc-LacZ*, *Hh-lacZ* have been described (Chen Y. and Struhl 1996, Methot and Basler 1999, Wang G. et al. 1999, Wang G. et al. 2000b). Gal4 driver lines *MS1096* and *actin-Gal4* have been described (Wang G. et al. 1999). RNAi stocks targeting HDACs or their interacting proteins were obtained from Vienna *Drosophila* RNAi Center or National Institute of Genetics of Japan. To construct CiGA, an Asp718-HpaI fragment encoding double HA-tagged Ci N-terminal fragment (aa 1-686) and a HpaI-XbaI fragment encoding Gal4 transactivation domain (aa 768-878) were fused and inserted between Asp718 and XbaI sites of *pUAST* vector (Brand and Perrimon 1993). Using PCR-based mutagenesis, internal deletions and truncations were further introduced to generate CiGA variants. Flag-Sufu has been described (Zhang Q. et al. 2006).

Imaginal disc staining

Standard protocols for immunostaining of imaginal discs were used (Jiang and Struhl 1995). Antibodies used for this study were rat anti-Ci antibody, 2A1 (Motzny and Holmgren 1995), mouse anti-Ptc antibody (DSHB), mouse anti-Myc antibody (9E10, Santa Cruz)), mouse anti-HA antibody (F7, Santa Cruz), rabbit anti-LacZ (Affinity Bioreagents), mouse anti-LacZ (Sigma).

Cell culture, transfection, immunoprecipitation and western blot analysis

Drosophila S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100mg/ml of streptomycin at 24^{o}C. Transfection was carried out by Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. Immunoprecipitation and western blot analysis were carried out using standard protocols as previously described (Zhang W. et al. 2005).

Luciferase assays

For *ptc-luc* reporter assays, cl-8 cells were transfected with 1ug *ptc-luc* reporter construct (Chen C. H. et al. 1999), 50ng RL-PolIII renilla construct in 12 well plates together with 0.5ug Ci or its variants in the absence or presence of 0.25ug Sufu. After 48 hr incubation, the reporter assays were performed using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed in triplicate using FLUOstar OPTIMA (BMG LABTECH).

CHAPTER IV

Multiple Ser/Thr-rich degrons mediate the degradation of Ci by the HIB E3 ubiquitin ligase

Introduction

The Cul3-based E3 ubiquitin ligases regulate several important developmental signaling pathways including Hh and Wnt pathways (Angers et al. 2006, Zhang Q. et al. 2006). In general, they employ a large family of BTB domain containing proteins as their target recognition components (Pintard et al. 2004), but how Cul3-based E3 ligases recognize their substrates is unknown and the specific degrons in their target proteins remain to be identified for individual BTB proteins.

In Hh pathway, the Cul3-based E3 ligase complex containing the Hh-induced MATH and BTB domain protein HIB (Rdx, dSPOP) has been shown mediating the degradation of the hyperactive full-length from of Ci (Ci^{A}), which serves as a negative feedback loop to fine-tune the Hh signaling output (Kent et al. 2006, Ou et al. 2007, Zhang Q. et al. 2006). Importantly, this Cul3-HIB regulatory circuit is conserved because Gli proteins such as Gli2 and Gli3 can be degraded by HIB when expressed in *Drosophila* and SPOP, the mammaline homolog of HIB, can functionally replace HIB to degrade Ci (Zhang Q. et al. 2006).

Here we intend to investigate the mechanism by which Cul3-based E3 ligases regulate their substrates via identifying and characterizing degrons that mediate Ci degradation by Cul3-HIB. We show that Ci employs multiple Ser/Thr rich motifs as HIB specific degrons. T*rans*- and *cis*- cooperativity among HIB binding sites are essential for binding and degradation of Ci by Cul3-HIB, which provides a mechanistic insight into how Cul3-HIB recognizes its substrates. Moreover, the identified HIB-degron

consensus has important implication on the genome-wide prediction of substrates for Cul3-based E3 ligases.

Results

The N-terminal conserved domain is not required for HIB-mediated degradation

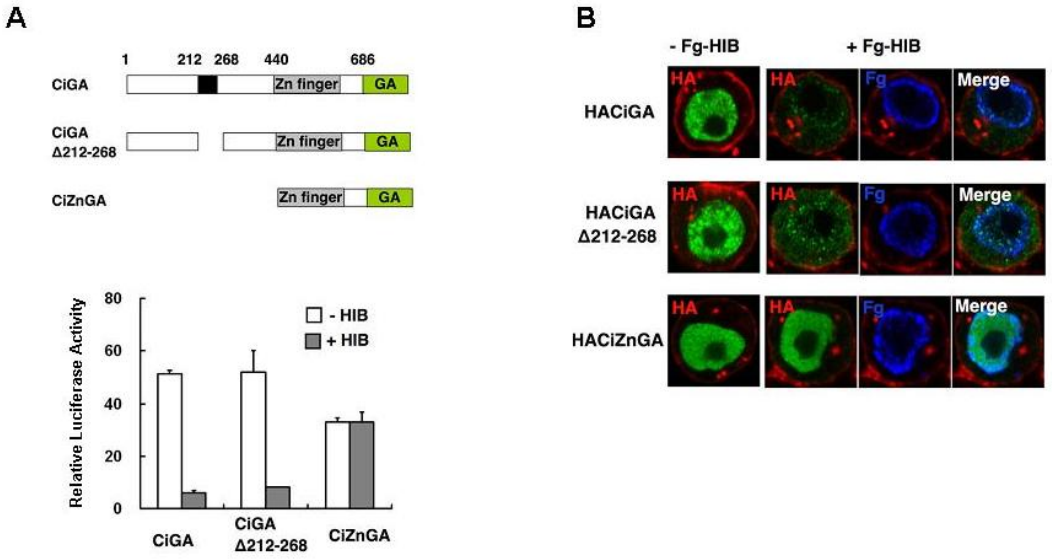


Figure 4.1 The N-terminal conserved domain is not required for HIB-mediated degradation. (A) Up: Diagrams of CiGA construct and its truncated forms. The black box represents the N-terminal conserved domain. Bottom: *ptc-luc* reporter assay in S2 cells transfected with the indicated Ci constructs in the absence or presence of a HIB-expressing construct. The *y* axis represents normalized *ptc-luc* activity. (B) S2 cells transfected with HA-tagged CiGA, CiGAΔ212-268 and CiZnGA either alone or together with Flag (Fg) -tagged HIB were immunostained with anti-HA antibody (Green), anti-Fg antibody (Blue) and phalloidin (Red), a dye labeling cell membrane.

Our previous study suggests that HIB targets Ci for degradation through both the N- (aa 1-440) and C- (aa 1160-1377) terminal regions of Ci (Zhang Q. et al. 2006). The N-terminal region of Ci contains a 49 aa domain NR (N-terminal regulatory element; aa209-258) that is conserved among all the

Ci/Gli family members and contains a destruction signal called D_N degron that targets Gli1 for degradation (Croker et al. 2006, Huntzicker et al. 2006). To determine if the D_N degron mediates the Ci degradation by HIB, we constructed a Ci-Gal4 fusion protein (CiGA) in which the N-terminal half of Ci (aa 1-686) including the Zn finger DNA binding domain was fused to the Gal4 activation domain (Fig. 4.1A). Expression of CiGA in S2 cells activated a *ptc-luc* reporter gene, and the activity of CiGA was blocked by coexpression of wild-type HIB but not a truncated HIB lacking the MATH domain (Fig. 4.1A; data not shown). A CiGA varaint lacking the D_N degron (CiGAΔ212-268) was still inhibited by HIB (Fig. 4.1A). By contrast, deleting the entire N-terminal domain (CiZnGA) abolished the HIB-mediated inhibition (Fig. 4.1A). By immunostaining, we found that CiGA, CiGAΔ212-268 and CiZnGA were all localized in the nuclei (Fig. 4.1B, column 1). Coexpression of HIB diminished the levels of CiGA and CiGAΔ212-268 but did not significantly affect the level of CiZnGA, suggesting that CiGA and CiGAΔ212-268 were degraded by HIB whereas CiZnGA was not (Fig. 4.1B, columns 2-4). Taken together, these results suggest that the D_N degron is dispensable for the HIB-mediated degradation of Ci.

***In vitro* function analysis of HIB binding sites**

Using a combination of yeast two hybrid, GST pull down and immunoprecipitation assays, Dr. Qing Zhang (Former postdoc in the lab) identified six major sites in Ci that mediate HIB binding (Fig. 4.2A). To determine the relative contribution of individual HIB binding sites to the regulation of Ci by HIB, we developed a cell-based assay in which we mutated HIB binding sites in the context of either CiGA or full-length Ci and measured their transcriptional activity in the absence or presence of HIB coexpression.

We initially focused on the N-terminal region of Ci. When expressed in S2 cells, CiGA activated a *ptc-luc* reporter gene but the activity was repressed by coexpression of HIB (Fig. 4.1A, 4.2C). Mutating all the four N-terminal HIB binding sites (CiGAm1-4) greatly diminished but not completely abolished the HIB-mediated repression (Fig. 4.2B, C). CiGA lacking S1 and S2 (CiGAm12) was repressed by HIB

similarly to the wild-type CiGA whereas CiGA lacking S3 and S4 (CiGAm34) was resistant to the HIB-mediated inhibition similarly to CiGAm1-4 (Fig. 4.2B, C). Collectively, these results suggest that S3 and S4 are the critical N-termianl sites in Ci degradation by HIB. Mutating either S3 or S4 in the context of CiGA (CiGAm3 or CiGAm4) or CiGAm12 (CiGAm123 or CiGAm124) did not significantly affect the HIB-mediated repression (Fig. 4.2B, C), suggesting that S3 and S4 act redundantly in the context of CiGA to mediate HIB inhibition. Of note, CiGAm1-4 was still partially repressed by HIB coexpression, implying additional weak HIB binding site(s) may exist in CiGA besides S1-4 (Fig. 4.2B, C).

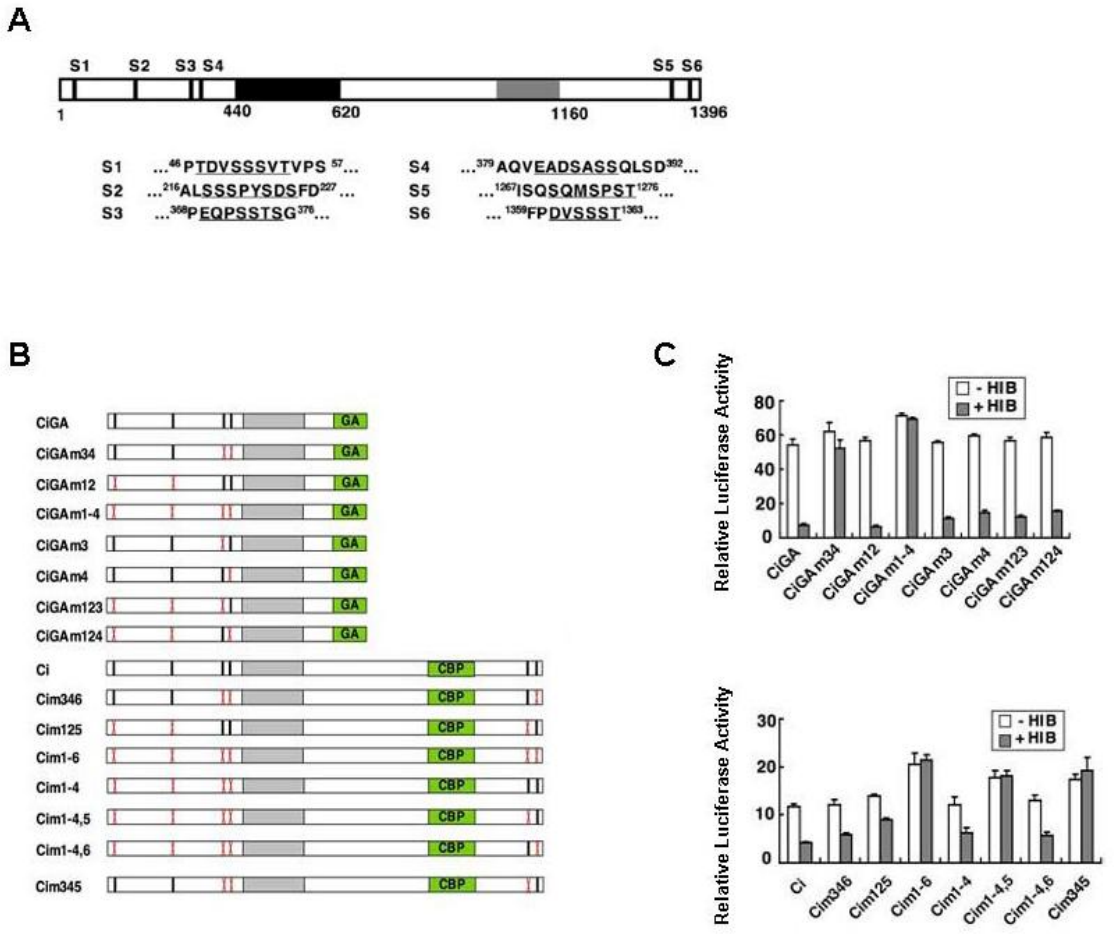


Figure 4.2 *In vitro* function analysis of HIB binding sites. (A) Diagrams of the full-length Ci with the 6 HIB-binding sites (S1 to S6) indicated by individual bars and the sequence of individual sites shown underneath. (B) Diagrams of CiGA and full-length Ci constructs with wild type and mutated HIB-binding sites indicated by bars and crosses, respectively. (C) *ptc-luc* assays in S2 cells expressing indicated CiGA (up) or full-length Ci (bottom) constructs in the absence or presence of HIB coexpression. The *y* axis represents normalized *ptc-luc* activity.

We also examined the relative contribution of the C-terminal sites (S5 and S6) in the context of a full-length Ci lacking S1-4 (Cim1-4). When expressed in S2 cells, Cim1-4 activated a *ptc-luc* reporter but the activity was suppressed by HIB coexpression (Fig. 4.2B, C). Mutating S6 in the context of Cim1-4 (Cim1-4,6) did not significantly affect HIB inhibition whereas mutating S5 (Cim1-5) nearly abolished HIB inhibition, similar to mutating both S5 and S6 (Cim1-6), suggesting that S5 is the critical site in the C-terminal region (Fig. 4.2B, C). Both Cim346 and Cim125 were suppressed by HIB (albeit less effectively than the wild-type Ci), whereas Cim1–6 was resistant to the HIB-mediated inhibition (Fig. 4.2B, C). Consistent with S3/4 and S5 being critical for HIB binding, mutating these sites in the context of full-length Ci (Cim345) abolished the HIB-mediated inhibition (Fig. 4.2B, C).

Trans-cooperation of HIB binding Sites

Although S3/4 are the critical sites for HIB binding to the N-terminus of Ci, surprisingly, we found that CiGAΔ1-268, a fragment containing both sites, failed to response to the HIB-mediated repression in a *ptc-luc* assay (Fig. 4.3C) as well as failed to bind HIB in an immunoprecipitation assay (data not shown). Thus, the interaction between HIB and Ci N-terminus (aa 1-440) may require additional element(s) in the aa 1-268 of Ci.

We noticed that it has been reported that BTB domains in several BTB-ZF family members form homodimers (Espinas et al. 1999, Gao and Benyajati 1998), and the crystal structure of the BTB domain of PLZF revealed a tightly intertwined dimer with an extensive hydrophobic interface (Ahmad et al. 1998). Accordingly, a postdoc in our lab found that HIB exists as a dimer or oligomer through both N- and C-terminal regions in an immunoprecipitaion assay (data not shown). In addition, by FRET analysis and immunoprecipitation assay, we found that full-length Ci also form dimer (data not shown). Consistent with a previous observation that Gli proteins might form homodimers or heterodimers through an interaction between the first two Zn fingers (Nguyen et al. 2005), we showed that HA-Ci1-686 associated with Myc-Ci440-1160 (Fig. 4.3A). Nevertheless, we also observed that HA-Ci1-686 associated with

Myc-Ci1-440 (Fig. 4.3A), which does not contain the zin-finger DNA binding domain, implying the existence of an additional dimerization surface(s) in the N-terminal region of Ci (Fig. 4.3A). To map the N-terminal dimerization domain, a Flag-tagged Ci1-440 (Flag-CiN) was coexpressed with various Myc-tagged Ci N-terminal fragments in S2 cells, followed by immunoprecipitation analysis. Interestingly, Flag-CiN pulled down Myc-CiN, Myc-CiN Δ 212-268 and Myc-Ci1-212 but not Myc-Ci268-440 (Fig. 4.3B). Moreover, Flag-Ci1-212 interacted with Myc-Ci1-212 whereas Flag-Ci268-440 didn't interact with Myc-Ci268-440, suggesting the N-terminal region between aa 1-212 mediates dimerization (Fig. 4.3B). In other words, Ci268-440, which contains intact S3/4 but fails to response to HIB inhibition, is dimerization deficient.

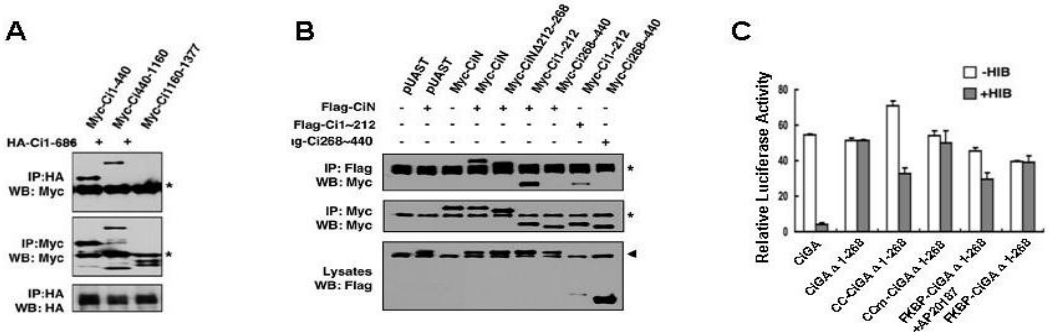


Figure 4.3 *Trans*-cooperativity between N-terminal HIB binding sites mediated by dimerization. (A) S2 cells were transfected with the indicated Ci constructs. Cell lysates were subjected to immunoprecipitation and western blot analysis with indicated antibodies. Ci1-686 interacted with N-terminal (Ci1-440) and medium (Ci440-1160) parts but not the C-terminal (Ci1160-1377) part of Ci. Asterisks indicate IgG. (B) S2 cells were transfected with indicated Ci constructs. Cell lysates were subjected to immunoprecipitation and western blot analysis with indicated antibodies. aa 1-212 mediates the dimerization of N-terminus of Ci. Asterisks indicate IgG, and arrowhead indicates a non-specific band. (C) A *ptc-luc* assay in S2 cells expressing indicated CiGA constructs in the absence or presence of HIB coexpression. Forcing dimerization of CiGAΔ1-268 either by adding a coiled-coil dimerization motif (CC) or by AP20187-induced dimerization of FKBP motif restored the HIB-mediated repression on CiGAΔ1-268. CCm is a mutant version of CC motif, serving as a dimerization-deficient control. AP20187 was added to cells 24 hours before collection to a final concentration of 100nM.

Given the fact that both HIB and Ci exist as dimers, we speculate that HIB may bind Ci through multivalent interactions such that two intrinsically weak binding sites in Ci could interact with two MATH domains in a HIB dimer to achieve high occupancy. In this scenario, the unexpected observation that Ci268-440 containing intact S3/4 but lacking dimerization ability failed to bind HIB raised an interesting possibility that S3/4 may cooperate *in trans* through Ci dimerziation to mediate efficient HIB binding (Fig. 4.4C). To determine if dimerization promotes HIB binding to S3/4, several heterologous dimerization motifs, including the GCN4 Leucine Zipper dimerization motif (CC and its mutant version CCm) (O'Shea et al. 1991) and two copies of an inducible dimerization motif (FK506 binding protein (FKBP)) (Spencer et al. 1993), were fused to CiGA Δ 1-268. Intriguingly, addition of the wild-type but not the mutant CC rendered CiGA Δ 1-268 responsive to the HIB-mediated repression (Fig. 4.3C). Similarly, a small molecule (AP20187) induced-dimerization of FKBP-CiGA Δ 1-268 allowed it to be partially inhibited by HIB (Fig. 4.3C). Taken together, dimerization allows S3/4 to cooperate *in trans* to bind HIB.

Cis-cooperation of HIB binding Sites

Another unexpected result was that, despite S6 exhibiting higher HIB-binding affinity than S5 in the GST pull down assay (data not shown), mutating S6 in the context of Cim1-4 (Cim1-4,6) had no significant effect on the HIB-mediated inhibition whereas mutating S5 or both S5 and S6 nearly abolished the HIB-mediated inhibition (Fig. 4.2C). One possible explanation is that S5 may cooperate with S6 *in cis* to bind a HIB dimer; and when S6 is mutated, S5 may cooperate with other weak binding sites in the vicinity. To identify other S/T motifs that might cooperate with S5 *in cis*, a series of C-terminally truncated CiC fragments (Fig. 4.4A) were generated and their interaction with HIB was examined by immunoprecipitation assay. Ci1160-1377, Ci 1160-1359, Ci1160-1336 and Ci1160-1306 still interacted with HIB whereas Ci1160-1287, although containing the intact S5, failed to bind HIB (Fig. 4.4A; data not shown). Thus, the S/T rich motif located between aa 1287-1306, $_{1284}$FSTVNMQPITTS$_{1295}$ (named w),

might cooperate with S5 *in cis* (Fig. 4.4A). Indeed, mutating w alone in the context of Cim1-4 (Cim1-4, w) had no effect on the HIB-mediated inhibition, consistent with the notion that S5 still cooperates *in cis* with S6; however, mutating both w and S6 in the context of Cim1-4 (Cim1-4, 6w) abolished the HIB-mediated inhibition, suggesting that S5 cooperates with either S6 or w *in cis* to bind HIB such that mutating both sites is required to eliminate the ability of S5 to response to the HIB-mediated inhibition (Fig. 4.4B).

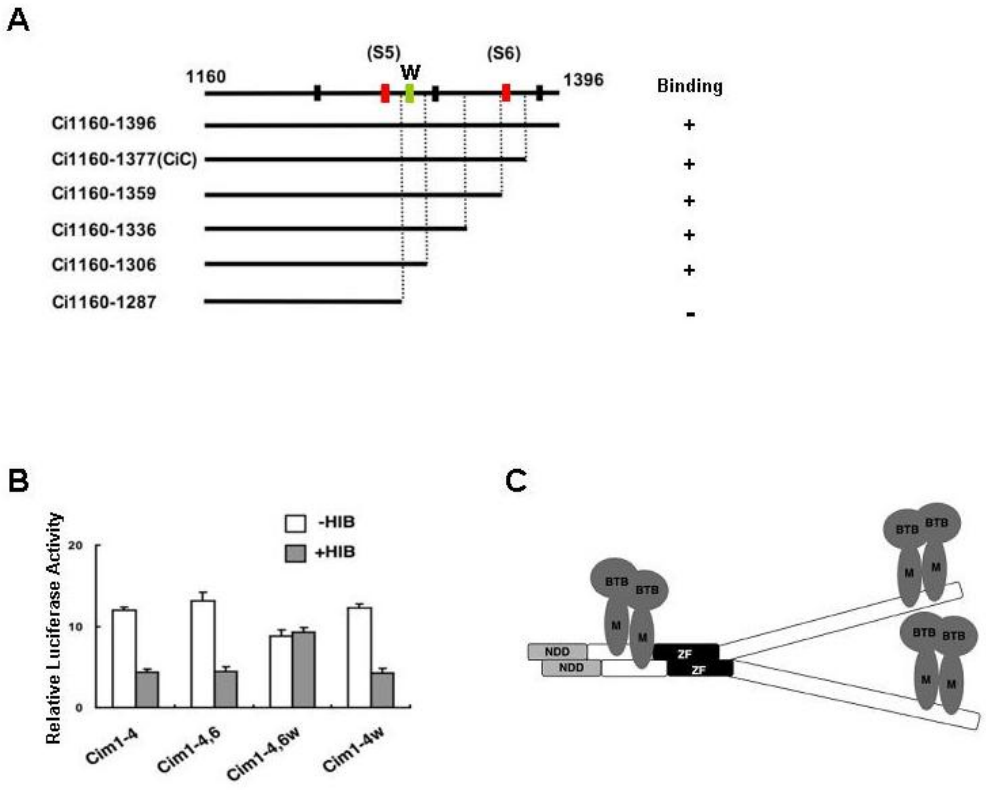


Figure 4.4 *Cis*-cooperativity among C-terminal HIB binding sites. (A) Diagrams of Ci C-terminal fragments with S/T rich motifs indicated by bars. Red bars represent S5/6, and green bar represents site w. (B) A *ptc-luc* assay in S2 cells expressing indicated full-length Ci or its mutant constructs in the presence or absence of HIB coexpression. The *y* axis represents normalized *ptc-luc* activity. (C) A model for multivalent interactions between HIB and Ci. NDD, N-terminal dimerization domain; ZF, Zn fingers; M, Math domain.

Discussion

In this study we intend to investigate the mechanism by which Cul3-based E3 ligases regulate their substrates via identifying and characterizing degrons that mediate Ci degradation by Cul3-HIB. Using a combination of yeast two hybrid, GST pull down and immunoprecipitation assays, Dr. Qing Zhang identified six major sites (S1-6) in Ci that mediate HIB binding. By applying a cell-based *ptc-luc* assay, I found two N-terminal sites (S3/4) and one C-terminal site (S5) being critical for HIB-mediated repression on Ci. Given that these are the first set of degrons identified for Cul3-based E3 ligases, our finding has important implications for the genome-wide prediction of substrates for Cul3-based E3 ligases.

The observation that both HIB and Ci form dimers raises a possibility that they engage in multivalent interactions such that intrinsically weak binding sites in Ci can bind cooperatively to HIB to achieve high occupancy. Indeed, we observed two types of cooperativity among HIB-binding sites in Ci. In the N-terminal region of Ci, S3/4 cooperate *in trans* through Ci dimerziation; whereas in the C-terminal region of Ci, S5/6/w cooperate *in cis* to mediate efficient HIB-binding. It is likely that the requirement of multiple sites that bind cooperatively to HIB provides a mechanism for regulating substrate specificity and binding affinity. Moreover, given the tendency of BTB domains to form dimers/oligomers (Espinas et al. 1999, Gao and Benyajati 1998), cooperative binding through multiple sites could be a general mechanism for Cul3/HIB/SPOP as well as other Cul3-based E3 ligases to recognize their substrates.

Another important observation is that all the indentified HIB-binding sites in Ci are S/T-rich motifs, some of which share a consensus containing 4 contiguous S/T residues preceded by one or more acidic residues (Fig. 4.2A). As we known, Ci becomes hyperactive but labile upon highest levels of Hh stimulation, and HIB E3 ligase plays a critical role in this Hh-induced Ci destabilization (Kent et al. 2006, Zhang Q. et al. 2006). Recent studies by our and other groups revealed that Ci/Gli transcription factors undergo activation-coupled phosphorylation in response to Hh stimulation, although little is known about

the responsible kinase(s) (Humke et al. 2010) (Fig. 2.6). Hence, it is possible that Hh may control Ci stablility by regulating HIB-Ci interaction through modulating the phosphorylation status of the S/T-rich HIB-binding sites in Ci. Interestingly, knocking down CK1 dramaticlly reduced the level of a processing-deficent form of Ci (Ci^U) in the presence of an active form of Fu (CC-Fu) that could mimic the situation of Hh activation (Fig. 4.5). Importantly, loss of HIB efficiently repressed the downregulation of Ci^U as well as ectopic *ptc* expression (Fig. 4.5). Thus, it is likely that a balance between active Fu-mediated Ci phosphorylation that is thought to promote HIB-Ci interaction and CK1-mediated Ci phosphorylation that probably attenuates HIB-Ci interation may tightly control the Ci stablilty upon Hh stimulation. In future, it would be very interesting to test this model by identifying the activation-coupled phosphorylation sites in Ci and examining if they overlap with HIB-binding sites, which will help us to reveal if substrate recognition by HIB/SPOP is regulated by phosphorylation.

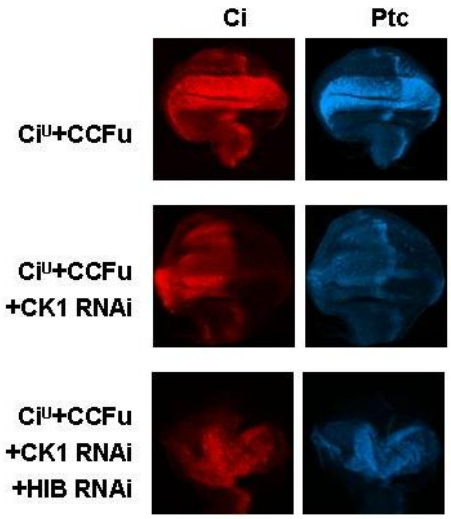


Figure 4.5 Loss of CK1 reduces Ci^U level in the presense of active Fu. *Drosophila* wing discs expressing indicated transgenes were immunostained to show the expression of Ci (red) and Ptc (blue). Loss of CK1 reduces the level of Ci^U and the ectopic *ptc* expression in the presence of CC-Fu, whereas loss of HIB restores Ci^U level as well as ectopic *ptc* expression.

Materials and methods

Plasmids

To construct CiGA, an Asp718-HpaI fragment encoding double HA-epitope tagged Ci N-terminal fragment (aa 1-686) and a HpaI-XbaI fragment encoding Gal4 activation domain (aa 768-878) were fused and inserted between Asp718 and XbaI sites of *pUAST* vector (Brand and Perrimon 1993). Using PCR-based mutagenesis, internal deletion, truncation and point mutation were further introduced to generate CiGA variants. To generate Ci truncation form constructs, cDNA fragments corresponding to different portions of Ci were amplified by PCR and subcloned in frame with six copies of myc-epitope tag or one copy of flag-epitope tag between BglII and Asp718 sites of *pUAST* vector. Point mutations as described in full length Ci were introduced by PCR-based site-directed mutagenesis. To generate FKBP-Ci chimeric protein constucts, a fragment encoding two copies of FKBP (FK506 binding protein with a single amino acid substitution, Phe36 to Val) was amplified from $pC_4M\text{-}F_v2E$ (a gift from ARIAD Pharmaceuticals; www.ariad.com/regulationkits) by PCR and subcloned between BglII and NotI sites of *pUAST* vector; then, NotI-Asp718 fragments encoding myc-CiN268-440 and HA-CiGA268-440 were inserted in frame after FKBP. To generate Leucine Zipper (CC)-Ci chimeric protein constructs, a peptide corresponding to the leucine zipper of the yeast transcriptional activator GCN4, (RMKQ**L**^{5}EDKVEE**L**^{12}**L**^{13}SKNYH**L**^{19}ENEVAR**L**^{26}KK**L**^{29}VGER) followed by a flexible linker (GSSG) was generated by multiple-step PCR and subcloned between BglII and NotI sites of *HA-pUAST* and *myc-pUAST* (O'Shea et al. 1991); then, NotI-Asp718 fragments encoding CiN268-440 and CiGA268-440 were inserted in frame after CC. As a control, a dimerization-deficient form of CC (CCm) (CC with two amino acid substitutions, Leu19 to Val and Leu26 to Val) was generated by PCR and fused to Ci and CiGA fragments as described above (Hu J. C. et al. 1990). Amino acid substitutions for various HIB binding sites and S/T-rich motifs were generated by PCR-based site-directed mutagenesis and verified by DNA sequencing.

Luciferase Reporter Assay

For luciferase reporter assays, S2 cells were cultured in 12 well plates and, for each well, transfected with 1ug *ptc-luc* reporter construct, 50ng RL-PolIII renilla construct (used as an internal control), together with 0.5ug Ci or its variant constructs in the absence or presence of HIB coexpression. After 48 hr incubation, the reporter assay was performed using the Dual-Luciferase reporter assay system (Promega) and measured using FLUOstar OPTIMA (BMG LABTECH).

Inducible dimerization system

To induce dimerization of FKBP-Ci fusion protein, AP20187 (a gift from ARIAD Pharmaceuticals; www.ariad.com/regulationkits) was added to cells 24hr after transfection to a final concentration of 100nM. Upon 24hr treatment, cells were harvested for Luciferase assay or Western blotting.

Cell culture, transfection, immunostaining, immunoprecipitation and western blot analysis

Drosophila S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100mg/ml of streptomycin at 24^{o}C. Transfection was carried out by Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. Immunoprecipitation and western blot analysis were carried out using standard protocols as previously described (Zhang W. et al. 2005). For immunostaining, S2 cells were harvested and washed with PBS, fixed with 4% formaldehyde at room temperature for 20 min, and incubated with the first antibody for 2 hours. Then, cells were washed three times with PBS followed by the secondary antibody staining for 1.5 hours. Antibodies used for this study are mouse anti-Flag (Sigma), mouse anti-HA (F7, Santa Cruz), rabbit anti-HA (F-11, Santa Cruz), and mouse anti-Myc (9E10, Santa Cruz).

CHAPTER V

Identification of a conserved PY-NLS in the N-terminus of Ci/Gli

Introduction

Despite functioning as a transcriptional activator in nucleus, the full length form Ci^F predominantly maintains a cytoplasmic inactive state due to its binding to the cytoplasmic complex containing the kinesin-related protein Cos2, the PEST-domain containing protein Sufu, and the Ser/Thr kinase Fu at microtubles (Monnier et al. 1998, Robbins et al. 1997, Sisson J. C. et al. 1997), as well as the rapid nuclear export mediated by a leucine-rich nuclear export signal (NES) located in its aa 703-835 (Wang Q. T. and Holmgren 1999). It has been shown that Hh signaling elevates the transcriptional activity of Ci^F partially via accelerating its nuclear import (Chen C. H. et al. 1999), but the underlying mechanism is largely unknown.

Since the nuclear pores prevent passive diffusion of macromolecules larger than ~40KDa (Bonner 1978, Paine et al. 1975), the nuclear localization of Ci^F (~155KDa) relies on active transportation, a process facilitated by members of the importin/karyopherin β (Kapβ) transport receptor superfamily. In general, Kapβs transport substrates through nuclear pore complexes via recognizing the nuclear localization signals (NLSs) in substrates, and then release them inside the nucleus upon binding the small GTPase RanGTP (Chook and Blobel 2001, Conti and Izaurralde 2001, Gorlich and Kutay 1999, Weis 2003). Previously, Holmgren's group identified a classic bipartite NLS (NLS^C) located at the end of the Zn finger domain of Ci (Wang Q. T. and Holmgren 1999). Later, however, the same group showed that mutating this NLS^C did not block the nuclear import of Ci upon the treatment of leptomycin B (LMB), a pharmacologic inhibitor blocking CRM1-dependent nuclear export (Sisson B. E. et al. 2006), which thus raised a possibility of the presence of an additional NLS within Ci.

Here using immunostaining, we found that the N terminus of Ci (CiN, aa 1-440) efficiently located in the nucleus independent on the previously identified NLS^{C}. By sequence analysis and mutagenesis study, we identified a functional PY-NLS (NLS^{PY}) in the N-terminal conserved domain of Ci. We showed that the NLS^{PY} functions in parallel with NLS^{C} in regulating the cellular localization and activity of Ci, and is conserved in members of Gli family proteins. Furthermore, we investigated the possible role of Transportin (Trn) and its relationship with Sufu in regulating Ci localization through NLS^{PY}, which will shed lights on our understanding how Hh regulates Ci activity through modulating its nucleocytoplasmic transportation.

Results

Identification of a second NLS in Ci N-terminus

Holmgren's group indentified the first NLS (NLS^{C}) in Ci, which is a typical bipartite NLS with the first basic cluster (aa 596-600) lying within the last Zn finger while the second one (aa 611-614) separated from the last Zn finger by 6 non-conserved amino acids (Fig. 5.1A) (Wang Q. T. and Holmgren 1999). However, we found that a Myc-tagged Ci N-terminal fragment containing aa 1-440 (Myc-CiN) efficiently localized in the nucleus though it does not contain the NLS^{C} and encodes a protein molecule exceeding the passive diffusion restriction of nuclear pore complexes, suggesting that the CiN may contain another NLS (Fig. 5.1B).

By sequence analysis, we found a putative PY-NLS-like sequence (aa 197-222), which fits the consensus "**Basic/hydrophobic motif**-X7~12-**R/K/H**-X2~5-**PY/L**" (Lee B. J. et al. 2006), locating near the N-terminal highly conserved domain of Ci (Fig. 5.1A). Strikingly, either deleting or mutating the C-terminal core ("**RKR**ALSS**PY**") of the PY-NLS-like sequence in Myc-CiNΔ212-268 or Myc-$CiNmNLS^{PY}$ respectively dramatically reduced the nuclear localization of Myc-CiN, indicating that the putative PY-NLS-like sequence is required for the nuclear localization of Myc-CiN (Fig. 5.1A-B).

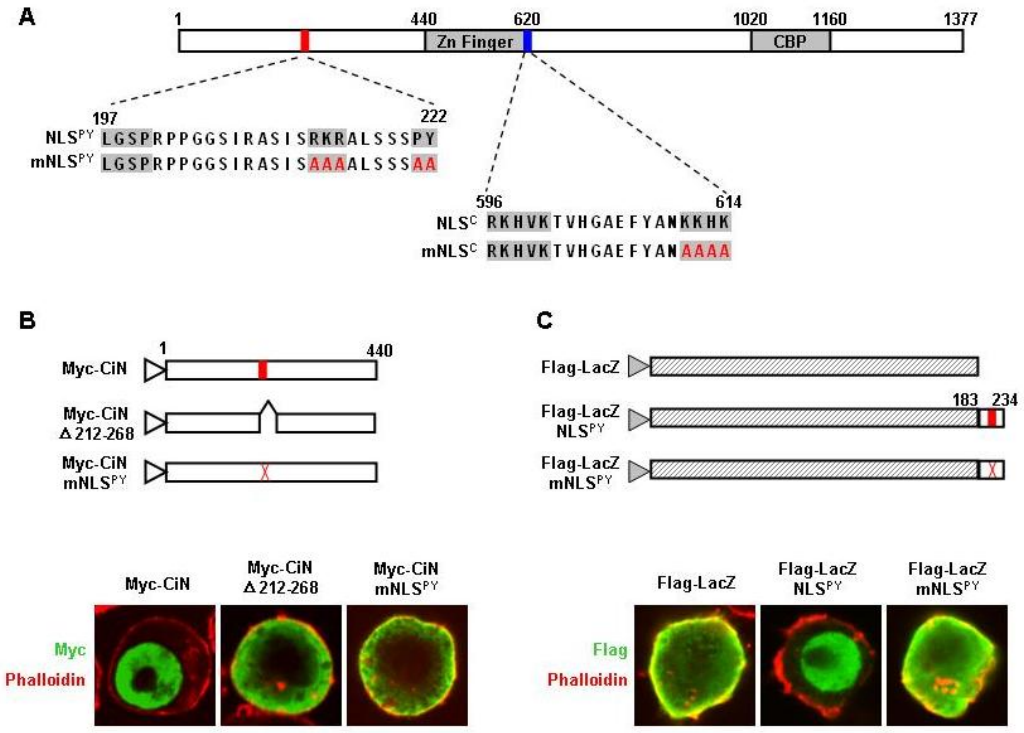


Figure 5.1 The N-terminal region of Ci contains a PY-NLS. (A) A diagram of Ci with gray boxes indicating Zn finger DNA-binding domain and CBP trans-activation domain. A red bar represents the newly identified PY-NLS with its wild-type (NLS^{PY}) and mutant ($mNLS^{PY}$) sequences listed below. A blue bar represents the classic bipartite NLS previously identified by Holmgren's group with its wild-type (NLS^{C}) and mutant ($mNLS^{C}$) sequences listed below. (B) Up: Diagrams of Myc-CiN with its internal deletion and NLS^{PY} mutant (indicated by a red cross) variants. Bottom: S2 cells transfected with Myc-CiN, Myc-CiNΔ212-268, and Myc-CiN$mNLS^{PY}$ were immunostained with anti-Myc antibody (Green) and phalloidin (Red), a dye labeling cell membrane. (C) Up: Diagrams of Flag-LacZ and Flag-LacZ-NLS chimeric proteins. Aa 183-234 including wild-type or mutant form of NLS^{PY} of Ci were fused C-terminally to the Flag-LacZ. Bottom: S2 cells transfected with Flag-LacZ, Flag-LacZNLS^{PY}, and Flag-LacZ$mNLS^{PY}$ were immunostained with anti-Flag antibody (Green) and phalloidin (Red).

To determine whether the PY-NLS-like sequence is sufficient to mediate active nuclear import of an exogenous protein, we fused aa 183-234 containing the putative PY-NLS-like sequence to the C terminus of Flag-LacZ (Flag-LacZNLS^{PY}, Fig. 5.1C) and expressed them in S2 cells. While Flag-LacZ is

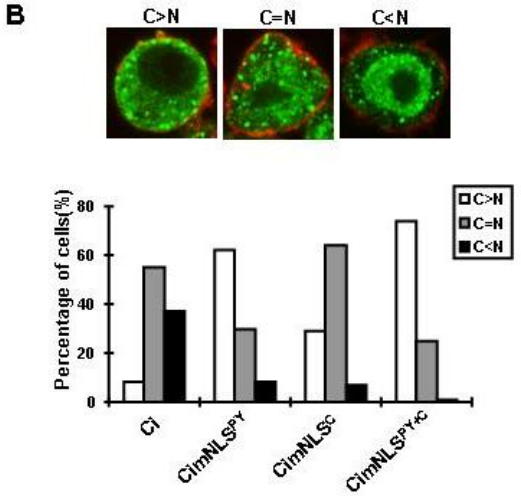
predominantly cytoplasmic, Flag-LacZNLS^{PY} exhibited strong nuclear staining (Fig. 5.1C). Moreover, fusing the same fragment but with the putative PY-NLS-like sequence mutated (Flag-LacZmNLS^{PY}) failed to promote the nuclear localization of Flag-LacZ (Fig. 5.1C). Taken together, we concluded that the N-terminal region of Ci contains a functional PY-NLS.

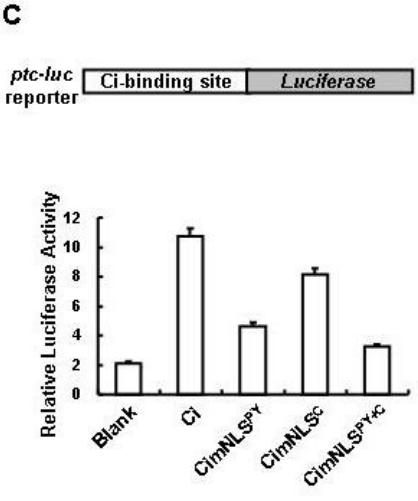
Ci localization and activity are regulated by its NLSs

To determine whether Ci localization is regulated by its NLSs, we mutated NLS^{PY} and NLS^{C} individually or in combination in full-length Ci background and expressed them in S2 cells (Fig. 5.2A). With 2 hours treatment of LMB, ~37% S2 cells transfected with wild-type Ci exhibited higher levels of signal in nucleus whereas less than 10% S2 cells transfected with CimNLS^{PY}, CimNLS^{C}, or CimNLS^{PY+C} exhibited higher levels of signal in nucleus (Fig. 5.2B), suggesting that mutating either NLS reduces the nuclear import of Ci. In addition, majority (~62%) of cells transfected with CimNLS^{PY} exhibited higher levels of signal in cytoplasm whereas majority (~64%) of cells transfected with CimNLS^{C} exhibited equal distribution between cytoplasm and nucleus (Fig. 5.2B), implying that NLS^{PY} has more effect on Ci nuclear import than NLS^{C} does in this scenario. Moreover, double NLS mutant (CimNLS^{PY+C}) showed further reduction of nuclear signal compared to single NLS mutants (Fig. 5.2B); thus, NLS^{PY} and NLS^{C} may function redundantly in promoting the nuclear import of Ci.

To determine the effect of NLSs in regulating Ci activity, we carried out a *ptc-luc* reporter assay in S2 cells. It is known that, in the absence of Hh stimulation, overexpressed wild-type Ci suffices to activate a *ptc-luc* reporter largely due to increased nuclear concentration of Ci (Chen C. H. et al. 1999, Wang Q. T. and Holmgren 2000) (Fig. 5.2C). Hence in this situation any defect in the nuclear import ability of Ci would reduce its nuclear concentration and consequencely decrease its activity. As expected, both CimNLS^{PY} and CimNLS^{C} exhibited reduced activity and CimNLS^{PY+C} showed the lowest activity, which is consistent with their severity of defect in nuclear import (Fig. 5.2B-C).


A
1
440
620
1020
1160
1377
Ci
CimNLS^PY
CimNLS^C
CimNLS^PY+C
Zn Finger
CBP


B
C>N
C=N
C<N
Percentage of cells(%)
0
20
40
60
80
Ci
CimNLS^PY
CimNLS^C
CimNLS^PY+C


C
ptc-luc reporter
Ci-binding site
Luciferase
Relative Luciferase Activity
0
2
4
6
8
10
12
Blank
Ci
CimNLS^PY
CimNLS^C
CimNLS^PY+C

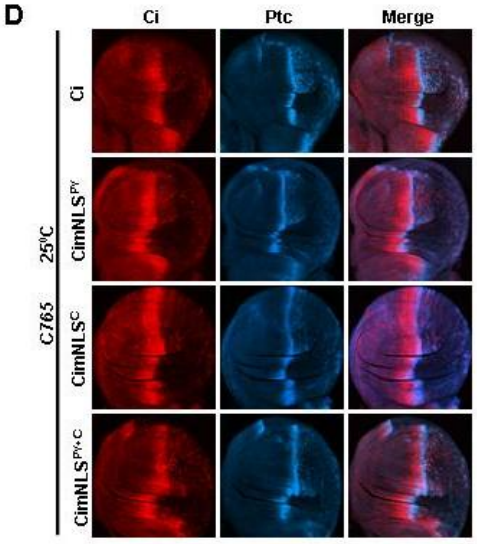

D
Ci
Ptc
Merge
C765
25°C
Ci
CimNLS^PY
CimNLS^C
CimNLS^PY+C

Figure 5.2 Ci localization and activity are regulated by its NLSs. (A) Diagrams of HA-Ci and its variants with NLS^{PY}, NLS^{C}, or both mutated. Red or blue bars represent wild-type NLS^{PY} or NLS^{C} respectively, whereas red or blue crosses represent mutated NLS^{PY} or NLS^{C} respectively. Gray boxes indicate Zn finger DNA-binding domain and CBP trans-activation domain. (B) S2 cells transfected with HA-Ci, HA-$CimNLS^{PY}$, HA-$CimNLS^{C}$, and HA-$CimNLS^{PY+C}$ were treated with 10ng/ml LMB for 2 hours before collection and immunostained with anti-HA antibody (Green) and phalloidin (Red). Up: Three cells represent different nucleocytoplasmic distributions of Ci. C>N: cells contain higher levels of Ci (or its variants) in cytoplasm than in nucleus. C=N: cells contain Ci (or its variants) equally distributed in cytoplasm and nucleus. C<N: cells contain higher levels of Ci (or its variants) in nucleus than in cytoplasm. Bottom: For each construct, a total of 100 cells were randomly picked and categorized based on the different nucleocytoplasmic distributions of HA signal. The *y* axis indicates the percentage of cells in each category. (C) Up: A diagram indicates *ptc-luc* reporter. Bottom: A *ptc-luc* reporter assay in S2 cells expressing Ci or its variants. The *y* axis represents normalized *ptc-luc* activity. (D) Wing discs expressing *UAS-Ci*, *UAS-CimNLS^{PY}*, *UAS-CimNLS^{C}*, *UAS-CimNLS^{PY+C}* with *C765* at 25^0C were immunostained to show the expression of Ci (Red) and Ptc (Blue).

To further determine whether mutations of NLS affect the activity of Ci *in vivo*, *UAS* transgenes expressing *UAS-Ci*, *UAS-CimNLS^{PY}*, *UAS-CimNLS^{C}*, *UAS-CimNLS^{PY+C}* were introduced into the 75B1 locus using the *phiC31* intergration system to ensure similar levels of transgene expression (Bischof et al. 2007). When expressed using a weak Gal4 driver, *C765*, wild-type Ci induced ectopic Ptc expression in P-compartment cells in response to Hh; whereas either singly or doubly mutating NLS remarkably reduced, albeit not completely abolished, the level of ectopic Ptc expression (Fig. 5.2D), consistent with both NLS^{PY} and NLS^{C} being required for the full activity of Ci.

Trn mediates CiN nuclear import through NLS^{PY}

The NLS-mediated nuclear import of substrate requires its being recognized by distinct members of Kapβ transport receptor superfamily. In contrast to the small classic NLS containing either a single or two clusters of basic residues recognized by the Kapα/Kapβ1 heterodimer (yeast Kap60p/Kap95p) (Conti

and Izaurralde 2001), the PY-NLS, consisting of a large linear signal with quite diverse sequences, is specifically recognized by Kapβ2 (yeast Kap104p) (Lee B. J. et al. 2006). There are two homologs of Kapβ2 in *Drosophila*, Trn (74% identity) and CG8219 (58% identity). To determine if they regulate Ci localization through the NLS^{PY}, we tested whether they are required for the nuclear localization of Myc-CiN, a simplified system that excludes the possible redundant effect of NLS^{C} and the opposite force of NES which otherwise would be included and could mask any subtle change in the full-length Ci background. To perturb the activity of Trn and CG8219, we performed RNAi to knockdown endogenous Trn or CG8219 in S2 cells transfected with Myc-CiN, and verified RNAi efficiency and specificity in S2 cells transfected with HA-Trn or HA-CG8219 since antibodies against endogenous Trn or CG8129 are unavailable. The N-terminal regions of Trn and CG8219 are thought functionally important by containing putative protein binding surfaces and two ARM domains but could introduce off-target effect since they are highly conserved between the two proteins, whereas C-terminal regions show more sequence diversity that allows knocking down individual protein specifically. Thus, we designed RNAi to target nonoverlapping N-terminal and C-terminal regions for each protein. As expected, Trn^{N} RNAi (targeting bp 100-800 of Trn) and $CG8219^{N}$ RNAi (targeting bp 100-800 of CG8219) knocked down both HA-Trn and HA-CG8219; whereas Trn^{C} RNAi (targeting bp 2100-2682 of Trn) and $CG8219^{C}$ RNAi (targeting bp 2100-2562 of CG8219) knocked down HA-Trn or HA-CG8219 respectively (Fig. 5.3A). Intriguingly, compared to Luci RNAi control which did not significantly affect the nuclear localization of Myc-CiN, all the dsRNAs knocking down Trn (including Trn^{N} RNAi, Trn^{C} RNAi, $CG8219^{N}$ RNAi) dramatically decreased the nuclear localization of Myc-CiN, but $CG8219^{C}$ RNAi that specifically targets CG8219 did not induce any obvious change (Fig. 5.3A). Therefore, Trn but not CG8219 plays a major role in promoting the nuclear import of Myc-CiN.

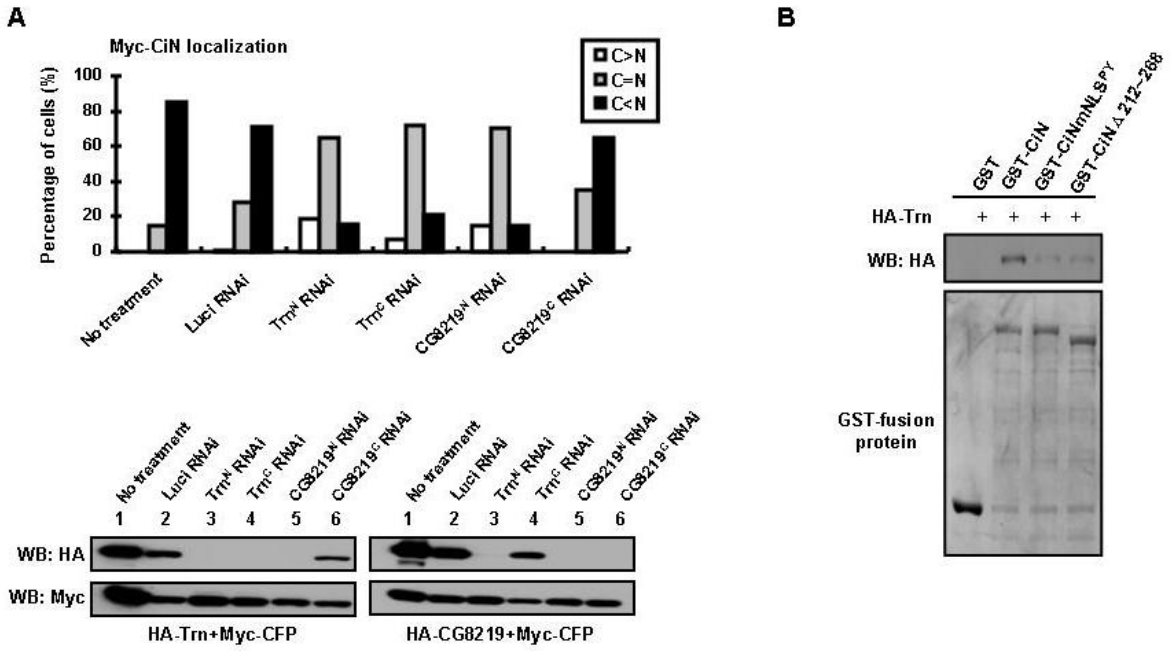


Figure 5.3 Trn mediates CiN nuclear import through NLS^{PY}. (A) Up: S2 cells pre-treated with indicated dsRNA were transfected with Myc-CiN and immunostained with anti-Myc antibody and phalloidin. Similar to Fig. 5.2B, 100 cells were randomly picked from each sample and categorized based on the different nucleocytoplasmic distributions of Myc signal. The *y* axis indicates the percentage of cells in each category. Bottom: S2 cells pre-treated with indicated dsRNA were transfected with HA-Trn or HA-CG8219 and Myc-CFP. Cell lysates were subjected to western blot analysis with indicated antibody. Myc-CFP served as an internal control for transfection efficiency. (B) Up: Similar amount of GST or GST fusion proteins containing CiN, CiNmNLS^{PY}, CiNΔ212-268 were incubated with cell extracts containing HA-Trn, followed by western blot analysis using anti-HA antibody. Bottom: Coomassie blue staining indicated the level of GST or GST fusion proteins.

We then examined the binding between Trn and CiN using GST pull down assay. GST-CiN but not GST protein alone bound to HA-Trn (Fig. 5.3B). Importantly, either deleting or mutating the C-terminal core ("**RKR**ALSS**PY**") of NLS^{PY} in GST-CiNΔ212-268 or GST-CiNmNLS^{PY} significantly reduced their binding affinity with HA-Trn, in line with the notion that Trn mediates the nuclear import of CiN through NLS^{PY} (Fig. 5.3B).

Cytoplasmic retention of Ci by Sufu involves masking NLS^{PY}

How Hh accelerates Ci nuclear import is largely unknown. It is known that Sufu has a role in sequestering Ci in cytoplasm (Monnier et al. 1998). Here we noticed that the Sufu binding motif locating in the N-terminal conserved domain of Ci is close to NLS^{PY} (Huntzicker et al. 2006)(Fig. 5.4A), raising interesting possibilities that Sufu binding may mask NLS^{PY}, and that Hh may promote Ci nuclear import by releasing Sufu binding and allowing NLS^{PY} to be recognized by Trn.

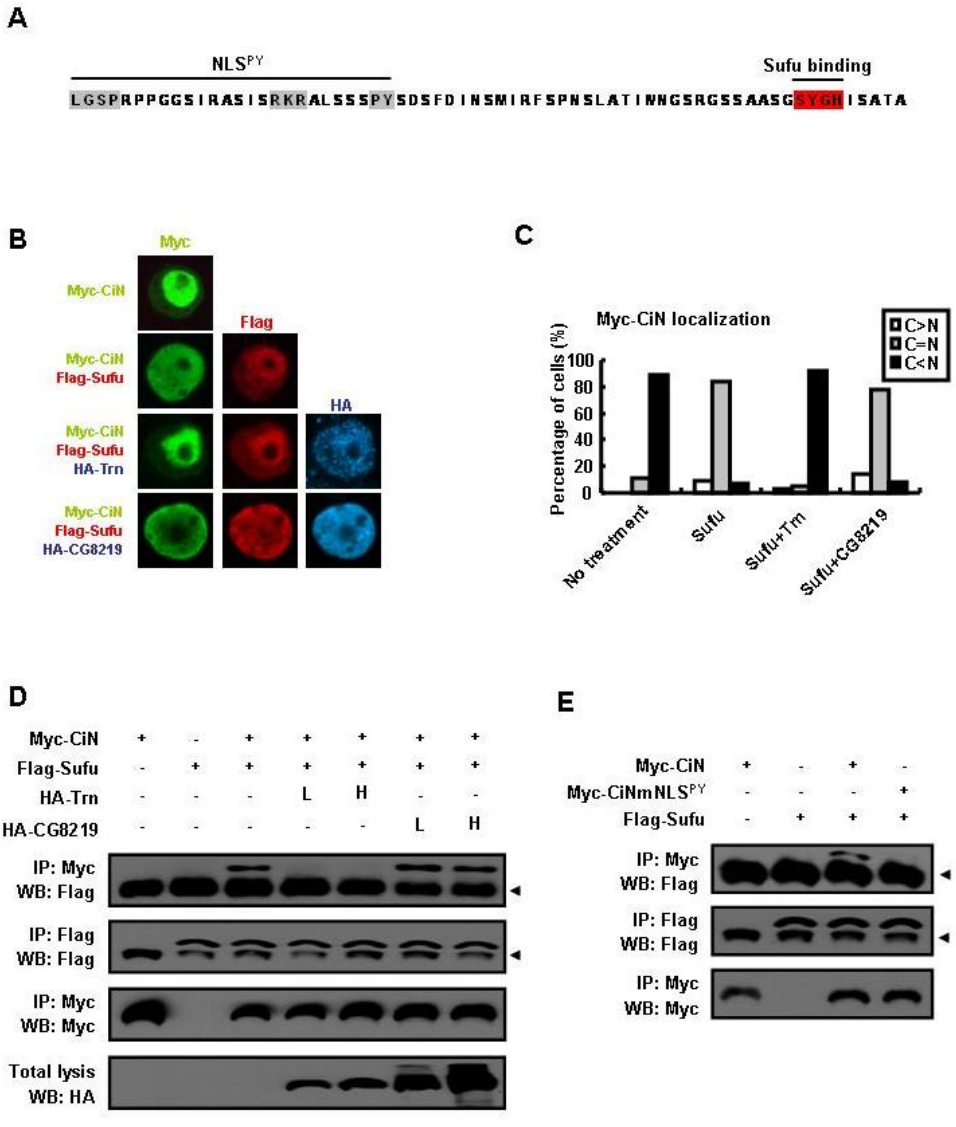


Figure 5.4 Cytoplasmic retention of Ci by Sufu involves masking NLS^{PY}. (A) A sequence indicates that the NLS^{PY} of Ci locates close to the previously identified Sufu-binding motif. Gray boxes represent the three energetically significant binding epitopes of NLS^{PY}. Red box represents the Sufu-binding motif. (B) S2 cells transfected with indicated constructs were immunostained with anti-Myc (Green), anti-Flag (Red) and anti-HA (Blue) antibodies. (C) Similar to Fig. 5.2B, 100 cells were randomly picked from each sample of (B) and categorized based on the different nucleocytoplasmic distributions of Myc signal. The *y* axis indicates the percentage of cells in each category. (D) S2 cells were transfected with fixed amounts of Myc-CiN and Flag-Sufu as well as increasing amounts of HA-Trn (lanes 4, 5) or HA-CG8219 (lanes 6, 7). Cell lysates were subjected to immunoprecipitation (IP) with indicated antibodies, followed by western blot (WB) analysis with indicated antibodies (top 3 panels). Cell lysates were also directly immunoblotted with anti-HA antibody (bottom panel). Arrowheads indicate IgG. (E) S2 cells transfected with indicated constructs were immunoprecipitated followed by western blot with indicated antibodies. Arrowheads indicate IgG.

As shown above, overexpressed Myc-CiN predominantly located in the nucleus of S2 cells (Fig. 5.1B, 5.4B-C), likely due to limited amount of endogenous Sufu to retain it in cytoplasm. Consistent with this, coexpressing Flag-Sufu redistributed Myc-CiN into cytoplasm (Fig. 5.4B-C). Interestingly, mutating NLS^{PY} dramatically reduced the binding between CiN and Sufu (Fig. 5.4E), implying NLS^{PY} being an essential interface for CiN/Sufu interaction. Furthermore, adding HA-Trn but not HA-CG8219 significantly restored the nuclear localization of Myc-CiN in the presence of Flag-Sufu (Fig. 5.4B-C), and also efficiently reduced the amount of Flag-Sufu pulled down by Myc-CiN (Fig. 5.4D), supporting our hypothesis that Trn competes with Sufu for binding NLS^{PY}.

The NLS^{PY} is highly conserved in Gli family proteins

To determine whether NLS^{PY} is conserved in Gli family proteins, N-terminal fragments of Gli proteins containing the putative PY-NLS-like sequences were fused to the C-terminus of Flag-LacZ. As shown in Fig.5.5B, Flag-LacZmouseGli2NLS^{PY} and Flag-LacZhumanGli3NLS^{PY} efficiently located in the nucleus of S2 cells; however, Flag-LacZfrogGli1NLS^{PY} failed to locate to nucleus (Fig. 5.5B).

Carefully sequence alignment revealed that although the C-terminal core of PY-NLS ("**R/K/H**-X2~5-**PY/L**") is conserved among all Gli proteins, the N-terminal hydrophobic motif presented in Ci, mouseGli2 and humanGli3 is absent in frogGli1 (Lee B. J. et al. 2006)(Fig. 5.5A). Hence, the NLS^{PY}-mediated nuclear transportation may be a conserved mechanism in regulating Gli2 and Gli3 but not Gli1 protein.

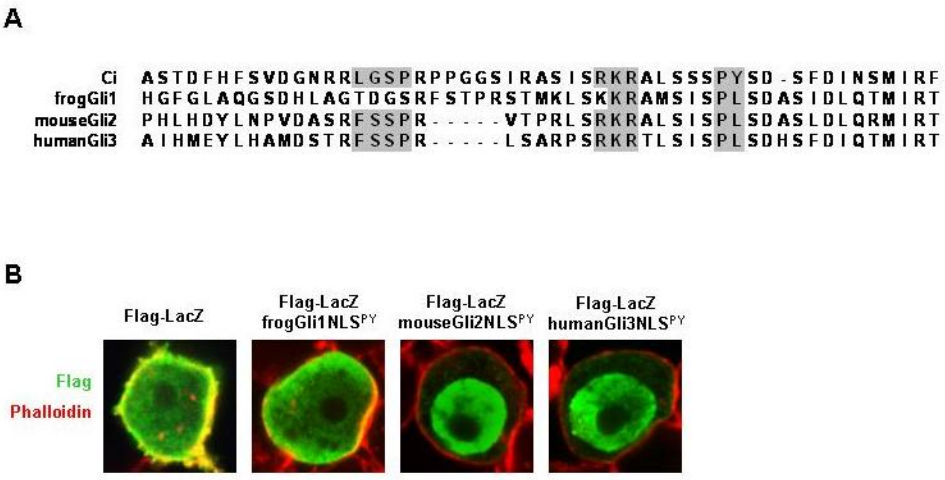


Figure 5.5 The NLS^{PY} is highly conserved in Gli family proteins. (A) Sequence alignments indicate that the PY-NLS-like sequence is highly conserved among Ci/Gli proteins, except that frogGli1 lacks the first hydrophobic motif. Gray boxes represent the three energetically significant binding epitopes of PY-NLS. (B) S2 cells transfected with Flag-LacZ, Flag-LacZfrogGli1NLS^{PY}, Flag-LacZmouseGli2NLS^{PY}, and Flag-LacZhumanGli3NLS^{PY} were immunostained with anti-Flag antibody (Green) and phalloidin (Red).

Discussion

Previous studies have suggested four criteria to verify a putative sequence as a functional NLS (Damelin et al. 2002). First, the sequence must be necessary such that deletion or mutagenesis of the sequence should mislocalize the substrate. Second, the sequence must be sufficient to target another protein into the nucleus. Third, the substrate containing the putative NLS must directly bind a specific Kapβ. Fourth, dysfunction in the corresponding Kapβ transport pathway should inhibit nuclear import of

the substrates. In this study, we demonstrated that a putative PY-NLS-like sequence located near the N-terminal conserved domain of Ci is required for the proper nuclear localization of CiN. Remarkably, this putative PY-NLS-like sequence is sufficient to mediate active nuclear import of a cytoplasmic LacZ protein. Furthermore, in line with the notion that a typical PY-NLS is specifically recognized by Kapβ2 (Lee B. J. et al. 2006), we detected a binding between CiN and Trn, one of *Drosophila* homologs of Kapβ2, in a GST pull down assay; and importantly, this binding is dependent on the intactness of the putative PY-NLS-like sequence. Finally, we showed that knocking down Trn by RNAi dramatically decreased the nuclear localization of CiN in S2 cells. Collectively, we conclude that the putative PY-NLS-like sequence in Ci is a functional NLS regulated by Trn transport pathway.

Unlike the classic monopartite or bipartite NLSs consisting of relatively small consensus with concentrated binding energy (Suel and Chook 2009, Wang Q. T. and Holmgren 1999), the PY-NLS is a type of large linear NLS containing at least three energetically significant binding epitopes: an N-terminal sequence-diverse basic/hydrophobic motif as Epitope 1, arginine, lysine, or histidine residue of the "**R/K/H**-X2~5-**PY/L**" motif as Epitope 2, and a "**PY/L**" motif as Epitope 3 (Lee B. J. et al. 2006). Each linear epitope is energetically quasi-independent, and the relative contribution of each one to the overall binding energy varies a lot among different PY-NLSs (Lee B. J. et al. 2006, Suel and Chook 2009). Specifially for the NLS^{PY} of Ci/Gli proteins, deleting or mutating highly conserved Epitopes 2&3 dramatically reduced the nuclear import of CiN, and consistently decreased the binding between CiN and Trn in a GST pull down assay. Nevertheless, those mutants did not completely abolish the binding with Trn; it is thus likely that Epitope 1 alone may still mediate some weak interaction with Trn although that is not enough to promote efficient nuclear import. In support of this notion, we found that the putative PY-NLS-like sequence in frogGli1 containing intact Epitopes 2&3 but lacking Epitope 1, which presents in the functional NLS^{PY} of Ci and all the other Gli proteins, does not behave like a functional NLS^{PY} as it failed to target a cytoplasmic LacZ protein into nucleus. Taken together, all three epitopes are

indispensable in this case such that Trn may bind them cooperatively to mediate efficient nuclear transportation of Ci/Gli proteins.

Given Ci containing more than one NLS, we assessed the relative contribution of the newly identified NLS^{PY} and the previously identified classic bipartite NLS^{C} in regulating the localization and activity of full-length Ci by mutating them individually or in combination. We found that mutating either of them reduced the nuclear signal of Ci in the presence of LMB, a drug that blocks CRM1-dependent nuclear export; while double NLS mutant exhibited a more dramatic reduction. Consistently, in a *ptc-luc* reporter assay in S2 cells, single NLS mutants showed less activity compared to wild-type Ci, and double NLS mutant exhibited the lowest activity. Moreover, both single and double mutants reduced the ability of Ci to ectopically activate Ptc in P-compartment cells in response to Hh when expressed in *Drosophila* wing discs using a weak Gal4 driver. Hence, both NLS^{PY} and NLS^{C} contribute to the nuclear import of Ci, and they appear to function redundantly to regulate Ci activity.

Finally, we explored the mechanism underling the regulation of NLS^{PY} in Ci. As we known, Ci is retained in cytoplasm by a microtuble-associated Cos2/Sufu/Fu complex in the absence of Hh (Monnier et al. 1998, Robbins et al. 1997, Sisson J. C. et al. 1997), while Hh somehow accelerates its nuclear import (Chen C. H. et al. 1999). Previously, Pascal Therond's group found that Hh-induced phosphorylation of Cos2 Ser572 resulted in its dissociation from Ci both *in vitro* and *in vivo* (Ruel et al. 2003, Ruel et al. 2007). Recently, our study on Fu kinase revealed that active forms of Fu induce phosphorylation and dissociation of Sufu/Cos2 from Ci (Fig. 2.6, data not shown). All these observations lead to an attractive model that the binding of Sufu/Cos2 may mask the NLSs of Ci in the absence of Hh, whereas the dissociation of Sufu/Cos2 from Ci by either Hh stimulation or active upstream component(s) may expose the NLSs, allowing Kapβs to bind and to import Ci into nucleus. Intriguingly, we noticed that the NLS^{PY} locates very close to the previously identified Sufu-binding motif in the N-terminal conserved domain of Ci (Fig. 5.4A). A remarkable reduction in the binding between $CiNmNLS^{PY}$ and Sufu suggested NLS^{PY}

indeed an essential interface for CiN/Sufu interaction. Importantly, we observed that adding Trn attenuated the Sufu-mediated cytoplasmic retention and significantly restored the nuclear localization of CiN, supporting our speculation that Trn competes with Sufu for binding NLS^{PY}. In future, a question to be answered is whether and how Hh switches the association of Ci from Sufu to Trn. Given the published and unpublished evidence that Hh induces phosphorylation of Sufu and Ci (Lum et al. 2003b) (Fig. 2.6) as well as the fact that the region containing NLS^{PY} is highly rich in Ser/Thr, it would be interesting to test if phosphorylation of that region is involved in Hh-mediated switch from Sufu to Trn. In addition, since the NLS^{PY} is conserved in some of Gli family proteins, it would also be interesting to determine whether mammals employ similar mechanism to regulate the nuclear transportation of Gli family proteins.

Materials and methods

Mutations and transgenes

To generate *UAS-Myc-CiN* construct, a fragment encoding Ci N-terminal fragment (aa 1-440) was amplified by PCR and inserted in frame between BglII and Asp718 sites of *pUAST-Myc* vector (Brand and Perrimon 1993, Tong and Jiang 2007). Using PCR-based mutagenesis, internal deletion and point mutations were further introduced to generate *UAS-Myc-CiN Δ212-268* and *UAS-Myc-CiNmNLS^{PY}*. To generate *UAS-Flag-LacZ-NLS* chimeric constructs, a NotI-Asp718 fragment encoding full-length LacZ amplified from *pcDNA3.1/V5-LacZ* (Invitrogen) and Asp718-XbaI fragments encoding regions including the putative PY-NLSs of Ci/Gli proteins (aa 183-234 of Ci, aa 54-106 of frogGli1, aa 201-247 of mouseGli2, and aa 265-312 of humanGli3) were fused and inserted between NotI and XbaI sites of *pUAST-Flag* vector (Brand and Perrimon 1993, Tong and Jiang 2007). Point mutations were further introduced into *UAS-Flag-LacZNLS^{PY}* to generate *UAS-Flag-LacZmNLS^{PY}* for Ci. To generate *UAS-HA-Ci*, an EcoRI-Asp718 fragment encoding three copies of HA-tag and an Asp718-XbaI fragment encoding Ci were fused and subcloned between EcoRI and XbaI sites of *pUAST* vector. Point mutations were then introduced by PCR-based mutagenesis to generate Ci variants including *UAS-HA-CimNLS^{PY}*, *UAS-HA-*

CimNLS^{C}, *UAS-HA-CimNLS^{PY+C}*. To generate *UAS-HA-Trn* and *UAS-HA-CG8219*, the coding regions of Trn and CG8219 were amplified by PCR and inserted in frame between BglII and Asp718 sites of *pUAST-HA* vector (Brand and Perrimon 1993, Tong and Jiang 2007). For GST pull down assay, DNA fragments encoding CiN, CiNmNLS^{PY}, and CiN Δ 212-268 were subcloned between BamHI and NotI sites of *pGEX4T3* vector. *UAS-Flag-Sufu* and *UAS-Myc-CFP* have been described previously (Zhang Q. et al. 2006, Zhang Q. et al. 2009). *C765* Gal4 driver has been described previously (Chen Y. et al. 2010). For generating flies with transgenes inserted at the 75B1 *attP* locus (Bischof et al. 2007), the coding regions for HA-tagged Ci variants were subcloned between EcoRI and XbaI sites of a modified *pUAST* vector with an *attB* sequence inserted upstream of the *UAS*-binding sites (Liu Y. et al. 2007).

Luciferase reporter assay

For Luciferase reporter assays, S2 cells were cultured in 12 well plates and for each well transfected with 1ug *ptc-luc* reporter construct, 50ng *RL-PolIII renilla* construct (an internal control), together with 0.5ug Ci or its variant constructs. After 48 hour incubation, the Luciferase reporter assays were performed using the Dual-Luciferase reporter assay system (Promega). Measurements for each sample were perfomed in triplicate using FLUOstar OPTIMA (BMG LABTECH).

Cell culture, transfection, immunostaining, immunoprecipitation and western blot analysis

Drosophila S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100mg/ml of streptomycin at 24^{o}C. Transfections were carried out by Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. Immunoprecipitation and western blot analysis were carried out using standard protocols as previously described (Zhang W. et al. 2005). For immunostaining, S2 cells transfected with indicated constructs and treated without or with 10ng/ml LMB for 2 hours were harvested and washed with PBS, fixed with 4% formaldehyde at room temperature for 20 minutes, and incubated with the first antibodies for 2 hours.

Then, cells were washed 3 times with PBS followed by the secondary antibody staining for 1.5 hours. Antibodies used for this study were mouse anti-Flag (M2, Sigma), rabbit anti-Flag (Thermo), mouse anti-HA (F7, Santa Cruz), rat anti-HA (3F10, a gift), and mouse anti-Myc (9E10, Santa Cruz).

RNAi in *Drosophila* S2 cells

DNA templates corresponding to Trn bp100-800, Trn bp2100-2682, CG8219 bp100-800, and CG8219 bp2100-2562 were generated by PCR and used to make dsRNA targeting N-terminus or C-terminus of Trn and CG8219 respectively. dsRNA targeting the Firefly Luciferase coding sequence was used as a control. All the dsRNAs were generated by MEGAscript High-Yield Transcription Kit (Ambion, #AM1334). For the RNAi knockdown experiments, S2 cells were first cultured in serum-free medium containing indicated dsRNA for 12 hours at 24^0C. After adding FBS to a final concentration of 10%, dsRNA-treated cells were cultured overnight before transfection with indicated constructs. After additional culturing for 2 days, cells were collected for immunostaining or western blot analysis.

GST pull down assay

GST-CiN fusion proteins and GST protein (control) were produced in E. coli and purified with glutathione agarose beads (gehealthcare). Then GST-CiN fusion protein or GST loaded beads were washed 3 times with ice-cold PBS containing 1% NP-40 and incubated with cell lysates derived from S2 cells expressing HA-Trn at 4^0C for 1.5 hours. After washing 3 times with cell lysis buffer, the beads were boiled in 1X SDS loading buffer, followed by western blot analysis.

CHAPTER VI

Summary and Future Directions

Using a combination of biochemical approaches and *Drosophila* genetic tools, this study intended to dissect the mechanisms underling the regulation of the Hh signaling pathway, one of the evolutionarily conserved signaling cascades essential for normal development as well as involved in multiple human diseases.

In the first part of this study, we were trying to answer how the Hh signaling transduces from the cell-membrane receptor system to the downstream transcription factor by modulating the status of intracellular signaling components. Previously, we observed that Hh activates Smo by inducing a conformational switch and clustering of its C-tails. Here through co-localization and FRET analysis both *in vitro* and *in vivo*, we found that Hh stimulation or Smo phosphorylation promotes the assembly of Smo-Cos2-Fu complexes as well as increases Fu-Fu intermolecular interactions. By fusing an exogenous dimerization motif, we showed that forced dimerization of Fu suffices to promote its activation. Interestingly, we found that Fu kinase dimerization or Hh stimulation induces multiple-site phosphorylation of its AL, and that phopho-mimetic mutations of Fu AL suffice to activate the Hh pathway. Finally, we demonstrated that activated Fu regulates Hh transcription factor, Ci, by inhibiting its proteolysis into a repressor form and by promoting its transcriptional activator activity through triggering phosphorylation of Ci, Sufu and Cos2, and interfering with Ci-Sufu and Ci-Cos2-kinase complex formation.

One puzzle raised by this study is that the CC-dimerized Fu didn't induce the highest level of pathway activity and less active than the phospho-mimetic mutants, especially in terms of its ability to block Ci processing. One possible explanation is that a negative regulator towards Fu AL phosphorylation, such as a Fu phosphatase, may exist such that the phospho-mimetic mutations which lock Fu in its "phosphorylation" state can overcome this inhibition and exhibit higher activity. Another possibility is that CC-induced dimerization does not fully mimic Hh-induced clustering of Smo-Cos2-Fu complexes such that additional modulation that cannot be achieved by CC-induced dimerization may be required for full activation of Fu. For example, Smo/Cos2 may function as a scaffold to recruit other kinase(s) to phosphorylate and futher activate Fu; and one promising candidate is CK1, which phosphorylates Fu C-terminal fragment (aa 306-805) in an *in vitro* kinase assay (data not shown), plays a positive role downstream of Smo in a genetic interaction analysis (Fig. 6.1A), and regulates the activity of phospho-mimetic forms of Fu (Fig. 6.1B-C). In line with this speculation, a recent study by Daniel Kalderon's group showed that mutating several putative CK1 sites enabled Fu^{EE} resistant to the regulation of CK1 RNAi as well as reduced the activity of a membrane-tethered mildly activated Fu (Zhou and Kalderon 2011), albeit further efforts remain needed to verify those sites indeed phosphorylated by CK1 biochemically. Finally, the inability of CC-Fu to induce high levels of Hh response could also attribute to the lack of activation of parallel pathway(s) that transduces Hh signal from Smo to Ci. Recent study has revealed that Smo can act as a canonical GPCR, which signals through Gαi to regulate Hh pathway activation (Ogden et al. 2008). Interestingly, we found that CC-Fu but not Fu synergized with active form of Gαi to activate Hh pathway (Table 6.1; data not shown). This additive effect could be simply due to the stabilization of Ci by active Gαi (Ogden et al. 2008) providing enough Ci^{F} for CC-Fu to activate; nevertheless, it remains possible that active Gαi may directly or indirectly impose extra promotion on the activity of CC-Fu. Given all the above possibilities, in future it would be interesting to identify additional negative or positive regulators for Fu and further clarify the underlying molecular mechanisms, which will help us to understand how Fu achieves its full activation.

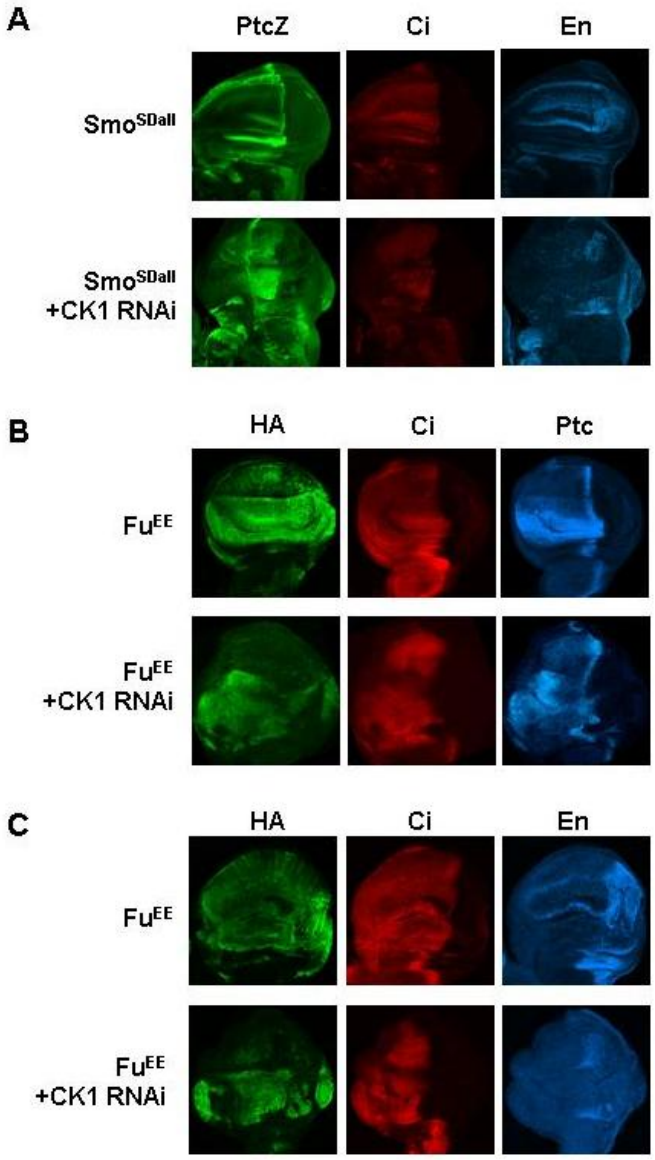


Figure 6.1 CK1 RNAi regulates the activity of Smo^{SDall} and Fu^{EE}. (A) Former result: *Drosophila* wing discs expressing Smo^{SDall} (a constitutively active phospho-mimetic form of Smo) without or with CK1 RNAi were immunostained to show the expression of PtcZ (Green), Ci (Red) and En (Blue). Knocking down CK1 dramatically attenuated the Smo^{SDall}-induced En expression. (B-C) *Drosophila* wing discs expressing HA-Fu^{EE} without or with CK1 RNAi were immunostained to show the expression of HA (Green), Ci (Red), Ptc (Blue in B) and En (Blue in C). Knocking down CK1 slightly reduced the Fu^{EE}-induced Ptc expression (B) while abolished the Fu^{EE}-induced En expression (C).

MS1096 (25^0C)	Female	Male
$G\alpha i^{Q205L}$	No phenotype	No phenotype
CC-Fu	Mild overgrowth	Mild overgrowth
$G\alpha i^{Q205L}$+*Fu*	No phenotype	No phenotype
$G\alpha i^{Q205L}$+*CC-Fu*	Severe overgrowth	Lethal

Table 6.1 CC-Fu synergizes with active Gαi to activate Hh pathway. Column 1: Indicated transgenes were expressed by *MS1096* wing-specific Gal4 driver at 25 degree. Columns 2&3: The adult wing phenotypes of female (column 2) or male (column 3) flies expressing indicated transgenes. $G\alpha i^{Q205L}$ is a consitutively active form of Gαi (Ogden et al. 2008). *MS1096* is located on the X chromosome and is expressed at higher levels in males than in females due to dosage compensation.

The second question this study didn't answer is how Hh initiates Fu activation. Our previous study revealed that Hh promotes phosphorylation, cell surface accumulation and C-tail dimerization of Smo requiring Fu kinase to phosphorylate Cos2 at Ser572 and in turn to antagonize the inhibitory effect of Cos2 (Liu Y. et al. 2007). Hence, Hh may elevate Fu kinase activity before its being dimerized by Smo C-tails. In other words, Hh may promote Fu activation via a dimerization-independent mechanism at the initial step of pathway activation. Of note, we found that coexpressing wild-type monomer form of Fu with Cos2 suffices to result in strong phosphorylation of Ser572 both *in vitro* and *in vivo* (Fig. 6.2; data not shown) but does not induce obvious ectopic activation of Hh target genes as CC-Fu does (Fig. 2.5A); thus, the Fu kinase activity achieved at the initiation step could be much lower than that induced by Smo C-tail dimerization. Until now, we still know little about the molecular mechanism for Hh initiating Fu activation. The observations that Fu C-terminal regulatory domain (aa 306-805) interacts with its N-terminal kinase domain (aa 1-305) (Fig. 6.3; data not shown) and functions as a domiant inhibitor of Hh pathway (Ascano and Robbins 2004, Ascano et al. 2002) and that deleting Fu C-terminus results in an enhancement of Fu kinase domain dimerization that could be abolished by re-adding Fu C-terminal

fragment (data not shown) lead us to the speculations that Fu may be subjected to auto-inhibition in the absence of Hh and that one possible mechanism to initiate Fu activation could be releasing its auto-inhibition by an Hh-triggered disruption of Fu intramolecular association. Interestingly, after narrowing down the binding domain (aa 477-643) in Fu C-terminus that interacts with Fu kinase domain (Fig. 6.3B), we noticed that it contains several putative CK1 sites (including Ser482, Ser485 and Thr486) which have been shown by Daniel Kalderon's group essential for the activity of a membrane-tethered mildly activated Fu (Zhou and Kalderon 2011). Therefore, in the future it would be very interesting to test whether Hh initiates Fu activation via inducing phosphorylation of its C-terminal regulatory domain by CK1 or other kinases and consequencely enhancing Fu kinase activity by releasing the auto-inhibition.

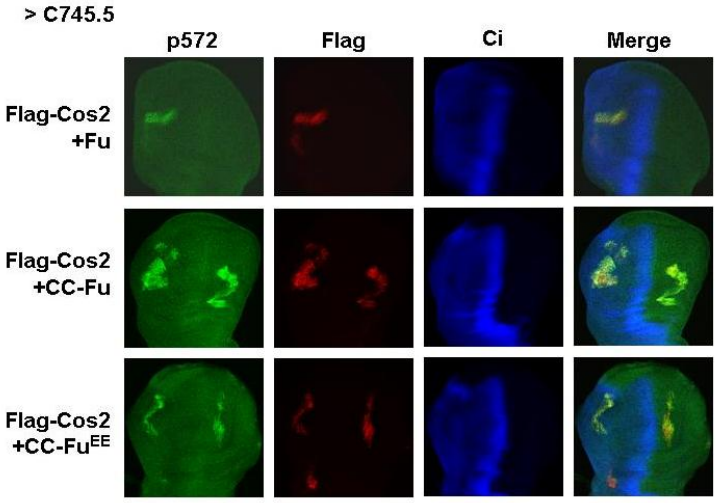


Figure 6.2 Wild-type monomer form of Fu induces phosphorylation of Cos2 at Ser572 similar to dimerization-activated Fu. *Drosophila* wing discs expressing Flag-Cos2 in clones using actin-Gal4 *C745.5* were immunostained to show the expression of Cos2 (Red), Ci (Blue) and the phosphorylation level of Cos2 Ser572 (Green). The phospho-antibody against Cos2 Ser572 was a gift from Dr. Pascal Therond. In the absence of Fu coexpression, the phosphorylation of Cos2 Ser572 occurs only in cells receiving Hh signal (Raisin et al. 2010) Coexpressing wild-type monomer form of Fu, CC-Fu or CC-Fu^{EE} ectopically induced similar levels of Cos2 Ser572 phosphorylation in A-compartment cells away from the A/P boundary without receiving Hh stimulation.

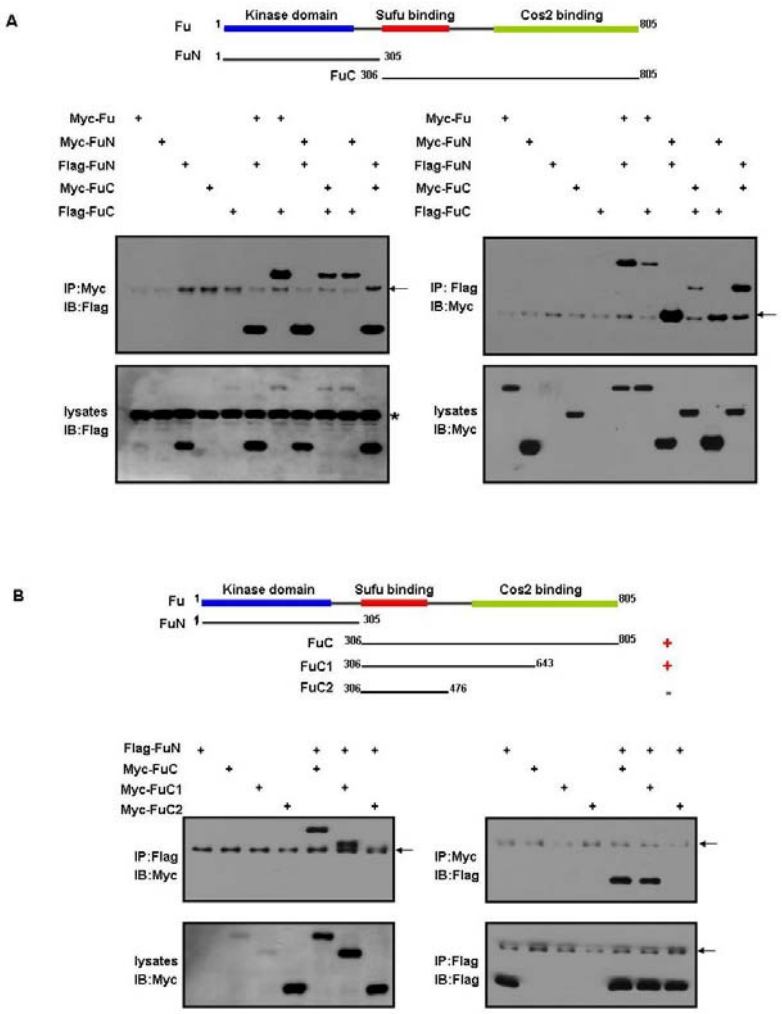


Figure 6.3 An intramolecular interaction between Fu C-terminal regulatory domain and Fu N-terminal kinase domain. (A) Up: Diagrams indicate Fu, Fu N-terminal kinase domain (FuN, aa 1-305) and Fu C-terminal regulatory domain (FuC, aa 306-805). Bottom: S2 cells were transfected with indicated constructs. Cell lysates were directly subjected to or first immunoprecipitated by indicated antibodies followed by western blot analysis. Full-length Fu bound both FuN and FuC, and FuN interacted with FuC. (B) Up: Diagrams indicate Fu, FuN, FuC, FuC1 (aa 306-643) and FuC2 (aa 306-476). FuN interacted with FuC, FuC1 (indicated by "+") but not FuC2 (indicated by "-"), implying that aa 477-643 is required for FuN/FuC intramolecular interaction. Bottom: S2 cells were transfected indicated constructs. Cell lysates were directly subjected to or first immunoprecipitated by indicated antibodies followed by western blot analysis.

Third, it remains a puzzle how Hh gradient is translated into differential Fu activity. On the one hand, using phospho-specific antibodies, we found that graded Hh signals and Smo activation progressively increase Fu AL phosphorylation (Fig. 2.5I-J), indicating that a gradient increase of Fu phosphorylation species may contribute to the elevation of pathway activity. On the other hand, we also observed a gradient increase of mobility shift of Fu in response to graded Hh treatments (data not shown), implying that, for one Fu molecule, more sites may get phosphorylated upon higher levels of Hh stimulation. In this scenario, it is possible that more sites in Fu AL get phosphorylated, but remains possible that other regions of Fu get phosphorylated. Given the previous cases that increasing phosphorylation in one kinase protein correlates with a stepwise increase in its kinase activity (Chong et al. 2001, Favelyukis et al. 2001), it is likely that the number of phosphorylated sites may also correlete with the strength of Fu kinase activity. Accordingly, we did observe that double phospho-mimetic mutations in Fu AL result in much higher Fu activity than single phospho-mimetic mutation does (Fig. 6.4). In future, we plan to use *in vitro* kinase assay or mass spectrometry to identify other phosphorylation sites in Fu, perform mutantgenesis analysis to evaluate their function, and eventually develop phospho-specific antibodies against single or multiple sites in combination to monitor the phosphorylation status of Fu in response to different levels of Hh, which will help us to determine how Hh induces differential phosphorylation of Fu kinase and whether that correlates with different pathway activity.

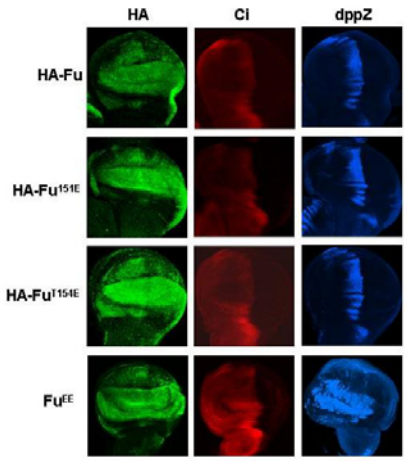


Figure 6.4 An intramolecular interaction between Fu C-terminal regulatory domain and Fu N-terminal kinase domain. *Drosophila* wing discs expressing *UAS-HA-Fu*, *UAS-HA-Fu^{T151E}*, *UAS-HA-Fu^{T154E}* and *UAS-HA-Fu^{EE}* using *MS1096* Gal4 driver were immunostained to show the expression of HA (Green), Ci (Red) and dppZ (Blue). Fu^{EE} induced strong ectopic activation of Hh target gene, *dpp*; whereas Fu^{T151E} and Fu^{T154E} barely induced any ectopic activation of *dpp* despite slightly reducing the level of Ci.

Finally, others' and our studies have revealed that either Hh or active forms of Fu induce phosphorylation of downstream components including Sufu, Cos2, and Ci (Fig. 2.6; data not shown); thus, it will be also worthwhile to identify and characterize the phosphorylation sites in them. We believe that modulating the phosphorylation status of those components by Hh could provide an important strategy for regulating the interactions among Sufu/Cos2/Ci/other cofactors, which eventually controls the balance between the generation of Ci^{R} and Ci^{A}.

In the remianing part of this study, we focused on investigating the regulation of the downstream Hh transcription factor, Ci. We identified and characterized three types of functional regulatory elements in Ci, including a transcriptional repression domain in the N-terminus of Ci, multiple Ser/Thr motifs serving as HIB/SPOP E3 ligase-specific degrons, and finally a novel NLS^{PY} near the N-terminal conserved domain of Ci.

As for the N-terminal repression domain of Ci, our preliminary data suggested that it contains multiple independent repression elements and likely recruits multiple corepressors. We believe that further effort to identify the possible corepressor(s) is a key to understand how the N-terminus of Ci exerts the repression ability. We plan to use a combination of biochemical purification and mass spectrometry methods to identify proteins binding to the N-terminus of Ci, from which a list of candidate corepressors can be generated. Then, we will either directly test whether knocking down the candidates in combination could result in derepression of CiGA that harbors the whole N-terminal repression domain,

or first map the specific binding motif(s) for a candidate followed by examining whether knocking down the individual candidate could lead to derepression of a corresponding CiGA truncation variant with the minimal N-terminal fragment that binds the candidate. We hope that the above strategies could overcome the issue of redundancy and give us clues on how the N-terminal repression domain of Ci works.

Then for the HIB/SPOP-specific degrons and NLS^{PY}, our study revealed that they are the essentinal interaction interfaces for Ci-HIB/SPOP and Ci-Trn respectively, and that a common feature of these two elements is locating in regions highly rich in Ser/Thr residues. It is known that the Hh-triggered degradation and nuclear import of Ci are at least patially mediated by HIB/SPOP E3 ligase and Trn respectively (Zhang Q. et al. 2006) (Chapter V). In addition, we recently observed that Ci undergoes activation-coupled phosphorylation in response to Hh stimulation or active forms of Fu (Fig. 2.6; data not shown). Thus, an interesting question to be answered is whether Hh regulates the stability and localization of Ci by modulating the phosphorylation status of HIB/SPOP-specific degrons or NLS^{PY} and correspondingly changing the binding affinity of Ci-HIB/SPOP or Ci-Trn. In future, we plan to identify the Hh-stimulated phosphorylation sites in Ci and also the responsible kinase(s). If we could find any phosphorylation sites overlapping with the HIB/SPOP-specific degrons or NLS^{PY}, we will perform mutagenesis analysis to determine whether the phosphoryaltion status of those sites affects the Ci-HIB/SPOP or Ci-Trn interaction and consequently influences the stability and localization of Ci.

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