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Posttranslational Regulation of Cubitus interruptus in Hedgehog Signaling in *Drosophila*

by

Brenda Ng

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Dedication

This work is dedicated to the memories of my mother's oldest brother, the gentle and patient Peter Chau, and her sister, Victoria Chau, who introduced me to *bun rieu*.

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I would like to thank my parents, Anthony Tai Hung Ng and Elaine Tran Ai Chau, for their love, patience, and support. Acknowledgements are also in order for the rest of my rather large family, especially my sister Cynthia, my uncles Davis and Ken and aunt Jennie for their advice, my uncle Charles for tolerating my temper, and my aunt Mary for the travails of traveling with me.

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Abstract

The program of spatial and temporal gene expression initiated upon reception of the Hedgehog (Hh) signal is regulated by the transcription factor Cubitus interruptus (Ci). Misregulation of Ci activity can result in developmental defects and cancer, and the Hh signaling pathway has evolved multiple layers of posttranslational regulation of Ci (such as repressor formation, protein degradation, and subcellular localization) to prevent both underactivation and hyperactivation of Hh target gene transcription and ensure proper patterning. The work described identifies and analyzes three distinct regulatory domains of Ci, using mutational analysis of transgenic Ci constructs and characterization with an *in vivo* wing disc assay and immunoblotting in the fruit fly, *Drosophila melanogaster*.

One domain, the RFD (Repressor Formation Domain), is required but not sufficient for Ci repressor (Ci-75) formation. The RFD is distinct from the site of cleavage and has homology to the vertebrate Gli proteins that are cleaved, Gli2 and Gli3. My results also support an alternative model for Ci-75 formation in which Supernumerary limb targets Smoothened for degradation, allowing cleavage of Ci by an exo-site protease.

A second domain, comprising residues 1160-1351, is downstream of the only identified activation domain in Ci, the CBP interaction domain. Using both tissue culture and *in vivo* expression in *Drosophila* I show that this region is involved in destabilizing Ci

protein, regulating Ci protein turnover, and thereby preventing its transformation into Ci activator (Ci-act).

The third domain, consisting of residues 1352-1398, has significant homology to Gli2 and Gli3, and is called the Gli Homology Domain (GHD). Using clonal analysis in the *Drosophila* wing disc and staining in salivary glands, I demonstrate that the GHD is crucial for both Ci activation and nuclear export.

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Chapter 1

Introduction

Developmental biology is the study of the processes by which a single cell becomes a multicellular organism. Processes necessary for the complete and correct formation of an organism include cell growth, cell division, apoptosis, pattern formation, cell fate determination, differentiation, axis specification, and morphogenesis. Development is not restricted to embryos as organisms continue to develop after birth, so the study of development also contributes to the fields of immunology, aging, and adult diseases such as cancer, as well as congenital disease and stem cells. A multitude of decisions confronts every cell during development, and each cell must respond correctly to cues that variously instruct cells to grow, proliferate, survive, and die. For example, during pattern formation, each cell in a field of cells must identify its position relative to its neighbors. This positional information plays a crucial role in axis specification, cell fate determination, differentiation, and ultimately the formation of tissues and organs.

Historically, classical developmental studies focused on embryology and were primarily descriptive in nature. J. E. Sulston's complete lineage map of the nematode *Caenorhabditis elegans*, which demonstrates the complexity of the events that give rise to a multicellular organism (Sulston et al., 1983), is a modern example of this type of approach. In a precursor to this lineage map, early embryologists mapped the early cell divisions of a

fertilized egg and observed that after the 16-cell stage, the embryo becomes asymmetric as cells individuate and begin to specialize. Using techniques such as dissociation and reaggregation of embryos, tissue grafts, and explants to perturb embryonic development, embryologists attempted to answer basic questions about the processes of development, such as whether different regions of the embryo develop independently from one another or whether development requires communication between cells. In a seminal series of transplantation experiments in 1923, Hans Spemann and Hilde Mangold demonstrated that cell fate could be determined by signals received from other cells in an embryonic organizer (translated in Spemann and Mangold, 2001). Biochemical and genetic studies have only recently identified the molecular nature of the signals produced by the Spemann organizer (reviewed in De Robertis, 2006).

At the same time that Spemann and Mangold were demonstrating that a transplanted dorsal blastopore lip could neuralize host tissue, Thomas Hunt Morgan and his students were using populations of the fruit fly *Drosophila melanogaster* to study the genetic basis for physical traits such as eye color. Scientists with backgrounds in both embryology and genetics began to use fruit flies as a model system to isolate mutants with visible defects in development (Waddington, 1939). For example, the subject of this thesis, the gene *cubitus interruptus* (*ci*), was first identified in such a manner because homozygotes with the recessive allele ci^1 have one or more distal interruptions in the L4, or cubital, wing vein in *Drosophila* (Tiniakow and Terentieva, 1933).

Developmental genetic studies have now identified many genes that regulate the developmental processes described by classical embryologists and have begun to elucidate the signal transduction pathways controlling development. One example is the Hox gene cluster, a family of transcription factors that pattern the body axis, first studied by Edward B. Lewis in *Drosophila* (Lewis, 1978). Others come from the work of Christiane Nusslein-Volhard and Eric Wieschaus, who in 1980 published the results of a genetic screen in *Drosophila* describing mutations affecting early embryonic development (Nusslein-Volhard and Wieschaus, 1980). Two of the genes identified in this screen were *ci*, which encodes a zinc finger transcription factor, and *hedgehog* (*hh*), which encodes a secreted protein with organizer activity.

Hedgehog and Cubitus interruptus in development

ci and *hh* are segment polarity genes that are required for correct anterior/posterior (A/P) patterning of the embryonic segments (Nusslein-Volhard and Wieschaus, 1980) and the larval imaginal discs which give rise to the adult tissues of a fruit fly (Schwartz et al., 1995; Tabata et al., 1992b). Hedgehog is a secreted signaling protein and Ci is the only known transcription factor downstream of Hedgehog.

The vertebrate homologs of *ci*, the *gli* genes, were initially identified as proteins that were upregulated in human tumors known as glioblastomas (Kinzler et al., 1988). Mutations

in other genes in the Hedgehog signaling pathway, such as the membrane proteins *patched* (*ptc*) and *smoothened* (*smo*), have also been implicated in a variety of human cancers, including basal cell carcinomas, medulloblastomas, small cell lung carcinomas, and solid tumors of the digestive tract (reviewed in Kasper et al., 2006; Taipale and Beachy, 2001; Xie and Abbruzzese, 2003). Like *ci*, the *gli*s are important in vertebrate patterning and play a part in developmental processes such as floor plate differentiation (Ding et al., 1998) and digit formation in vertebrates (Shin et al., 1999). Vertebrate Hedgehog proteins play similarly crucial roles in limb morphogenesis (Riddle et al., 1993), specification of left-right asymmetry (Levin et al., 1995), and cell fate specification in the neural tube (Roelink et al., 1995). The Hedgehog signal transduction pathway was subsequently found to be essential in the development of a wide variety of tissues, including the head, hair, lungs, somites, and gut (comprehensively reviewed in McMahon et al., 2003).

In all of these developmental systems, Hh is secreted from a group of cells and spreads for distances of many cell diameters to provide positional information in a field of cells and to allow for the adoption of different cell fates. For example, the *Drosophila* wing imaginal disc is functionally subdivided into two compartments as illustrated in Figure 1. It has an anterior compartment that expresses *ci*, and a posterior compartment that expresses *engrailed* (*en*), which codes for a transcription factor that represses *ci* expression in the posterior compartment (Eaton and Kornberg, 1990). The anterior/posterior (A/P) border where the two compartments meet acts as an organizer that patterns the entire wing disc.

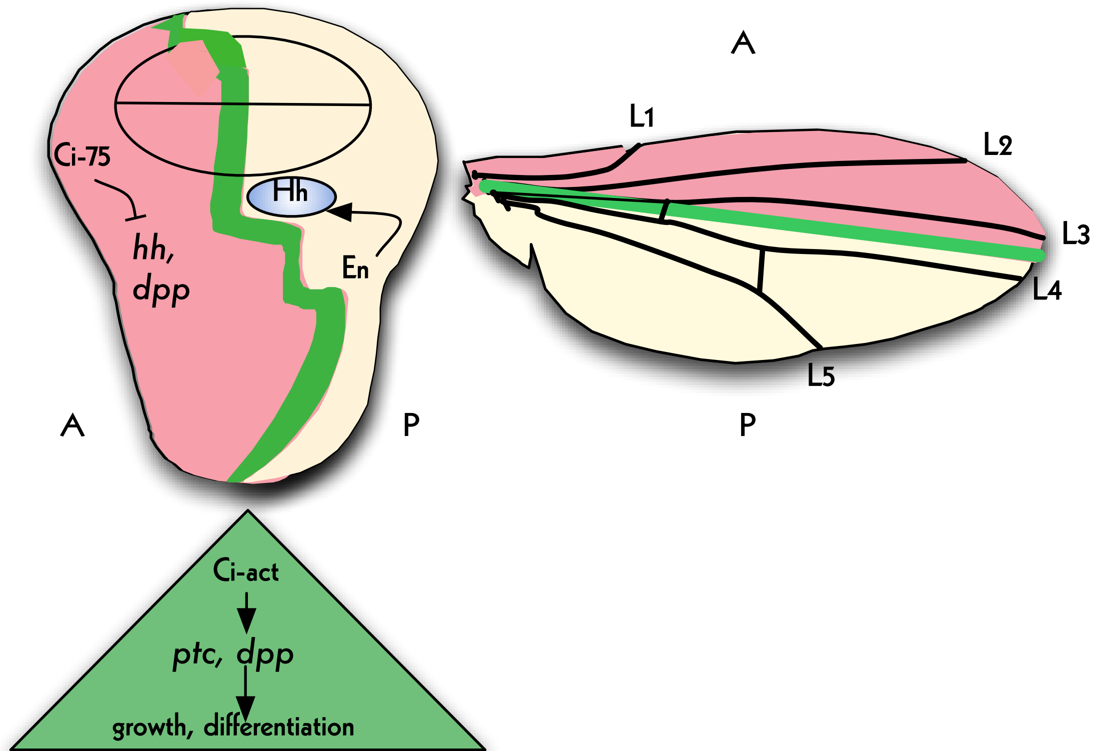


Figure 1: The A/P border and *cubitus interruptus* (*ci*) expression in the wing imaginal disc and adult wing. Red indicates areas of transcriptional repression and green indicates areas of transcriptional activation by Ci. The wing imaginal disc (left) is divided into a posterior (P) compartment marked by *engrailed* expression (in yellow) and an anterior (A) compartment that expresses *ci* (in red). In the far anterior of the wing disc away from the Hedgehog (Hh) signal, full length-Ci is cleaved to form Ci-75 which represses target genes such as *hh* and *decapentaplegic* (*dpp*). The A/P border where the two compartments meet (in green) acts as an organizer that patterns the entire disc. Hh is secreted by P cells and crosses the border, where it activates Ci. Activated Ci (Ci-act) turns on expression of Hh target genes such as *dpp* and *patched* (*ptc*). The pouch in the wing disc develops into the adult wing, diagrammed on the right, which is similarly organized.

Hedgehog protein, which has a N-terminal palmitic acid modification (Pepinsky et al., 1998) and a C-terminal cholesterol moiety (Porter et al., 1996; Porter et al., 1995), is extremely hydrophobic and its movement is therefore highly restricted. Hedgehog is produced in the posterior compartment, crosses the A/P border, and signals to anterior cells near the border. These anterior cells that receive the Hh signal express target genes such as *decapentaplegic* (*dpp*) (Domínguez et al., 1996), *collier* (*col*)/*knot* (*kn*) (Vervoort et al., 1999), and the putative Hh receptor *patched* (*ptc*) (Chen and Struhl, 1996). Secretion of the TGFβ homolog Dpp by anterior border cells is primarily responsible for long-range patterning and growth of the wing disc (Lecuit et al., 1996; Zecca et al., 1995).

Although many Hh targets in both invertebrates and vertebrates have yet to be identified, research has revealed that Hedgehog signaling, despite its apparently limited range, induces a variety of transcriptional responses. For example, absence of Hh in the far anterior of the wing disc leads to repression of *hh*, while intermediate levels result in *dpp* expression, and very high levels of Hh close to the A/P border cause *ptc* activation. One of the many unanswered questions regarding Hh signaling is how different levels of Hh can induce these distinctly different transcriptional responses in Hh receiving cells, especially since Ci is the only known transcription factor directly downstream of the Hh signal. Studies have shown that Ci is the transcriptional effector of Hh target genes such as *wingless* (*wg*) (Motzny and Holmgren, 1995), *col*/*kn* (Vervoort et al., 1999), *ptc* (Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993), *en,* and *dpp*, as well as *hh* itself (Domínguez et al., 1996; Méthot and Basler, 1999). Genetic analysis in *Drosophila* strongly suggests that there is no

Hh signaling without Ci and reports of Ci/Gli independent Hh signaling are more likely due to derepression of target genes in the absence of Ci repression rather than the existence of alternative Hh downstream transcription factors (Méthot and Basler, 2001).

Ci regulation of Hh target gene expression

Many aspects of Hh signal transduction, including how Hh regulates Ci and how a single transcription factor translates a gradient of Hh signaling into multiple transcriptional readouts, are still not well understood. It is known that Ci is modified at multiple points in this signaling pathway. For example, in the absence of Hedgehog, full-length 155 kilodalton (kD) Ci is cleaved to yield Ci-75, a shorter N-terminal fragment of Ci containing the zinc-finger DNA-binding domain and lacking a cytoplasmic localization domain and putative C-terminal activation domain (Aza-Blanc et al., 1997). Ci-75 translocates to the nucleus and acts as a transcriptional repressor of target genes, such as *hh* and *en*. In the presence of Hh, Ci is believed to function as a transcriptional activator (Ci-act) to up-regulate expression of target genes, such as *patched* (*ptc*) and *decapentaplegic* (*dpp*). The precise nature of this activator is unknown, although a domain has been identified in the C-terminal 1020-1160 amino acids of Ci which interacts with the transcriptional coactivator CREB Binding Protein (CBP) (Akimaru et al., 1997). However, unlike Ci-75, full-length Ci has not been detected in the nucleus without the use of drugs such as leptomycin B to block nuclear export (Chen et al., 1999a; Wang and Holmgren, 2000).

Ci cleavage and Ci's ability to activate and repress transcription bring an additional level of complexity to the analysis of how Ci regulates Hh target genes. Some genes, such as *hh*, appear to be targets only of the repressor Ci-75. However, some Hh targets, such as *ptc*, do not appear to be repressed by Ci-75. For example, the lack of *ptc* expression in Ci mutant clones and the ectopic expression of *ptc* in P cells ectopically expressing Ci-155 suggest that *ptc* expression is not simply repressed by Ci-75 in the absence of Hh (Domínguez et al., 1996). Instead, *ptc* expression appears to require some form of Ci that acts as an activator (Ci-act). Expression of a third set of Ci target genes, such as *dpp*, may not simply be controlled by the absolute presence or absence of a particular form of Ci. These targets appear to be regulated by the relative ratios of Ci activator to Ci repressor. Reports that *dpp* is ectopically activated in *ci* mutant clones suggest that Hh signaling at the border results in *dpp* activation primarily because formation of Ci-75 is blocked, thereby relieving transcriptional repression by Ci-75. However, ectopic expression of Ci-76 (a transgene expressing a 76kD form of Ci which acts a repressor and is believed to be analogous to Ci-75) at the border does not repress *dpp* expression (Méthot and Basler, 1999). Based upon these observations, it appears that *dpp* is a target of both the activator and repressor forms of Ci. Understanding how Ci is proteolysed to produce Ci-75, how Hh blocks this cleavage, and how Ci becomes a transcriptional activator are crucial to understanding how Hh acts to pattern a field of cells.

Hh signal transduction components & their effects on Ci function

The Hh signal transduction pathway is rather baroque with many identified components lacking clear roles, and it is certain that not all of its constituent components have been discovered. For example, the study of how the highly hydrophobic Hh moves and acts as a morphogen has identified no fewer than five proteins that play a role in this process: Ptc (Chen and Struhl, 1996), Dispatched (Burke et al., 2000), Tout velu (Bellaiche et al., 1998; The et al., 1999), Dally-like (Desbordes and Sanson, 2003), and Hedgehog interacting protein (Chuang and McMahon, 1999). The Hh signal transduction pathway is similarly complex and a basic model of what we now know about this pathway is presented in Figures 1 and 2. Using the *Drosophila* wing imaginal disc as a model system, the following paragraphs are a brief outline of Hh signal transduction with an emphasis on events and components pertaining to Ci.

The wing disc can be divided into two distinct developmental compartments, a posterior compartment marked by the expression of the homeobox containing gene *en* and an anterior compartment expressing *ci*. En represses *ci* expression in the posterior compartment, allowing Hh to be synthesized. The hydrophobic Hh protein then travels across the A/P compartment border and signals to anterior cells adjacent to the border (Eaton and Kornberg, 1990; Tabata et al., 1992a).

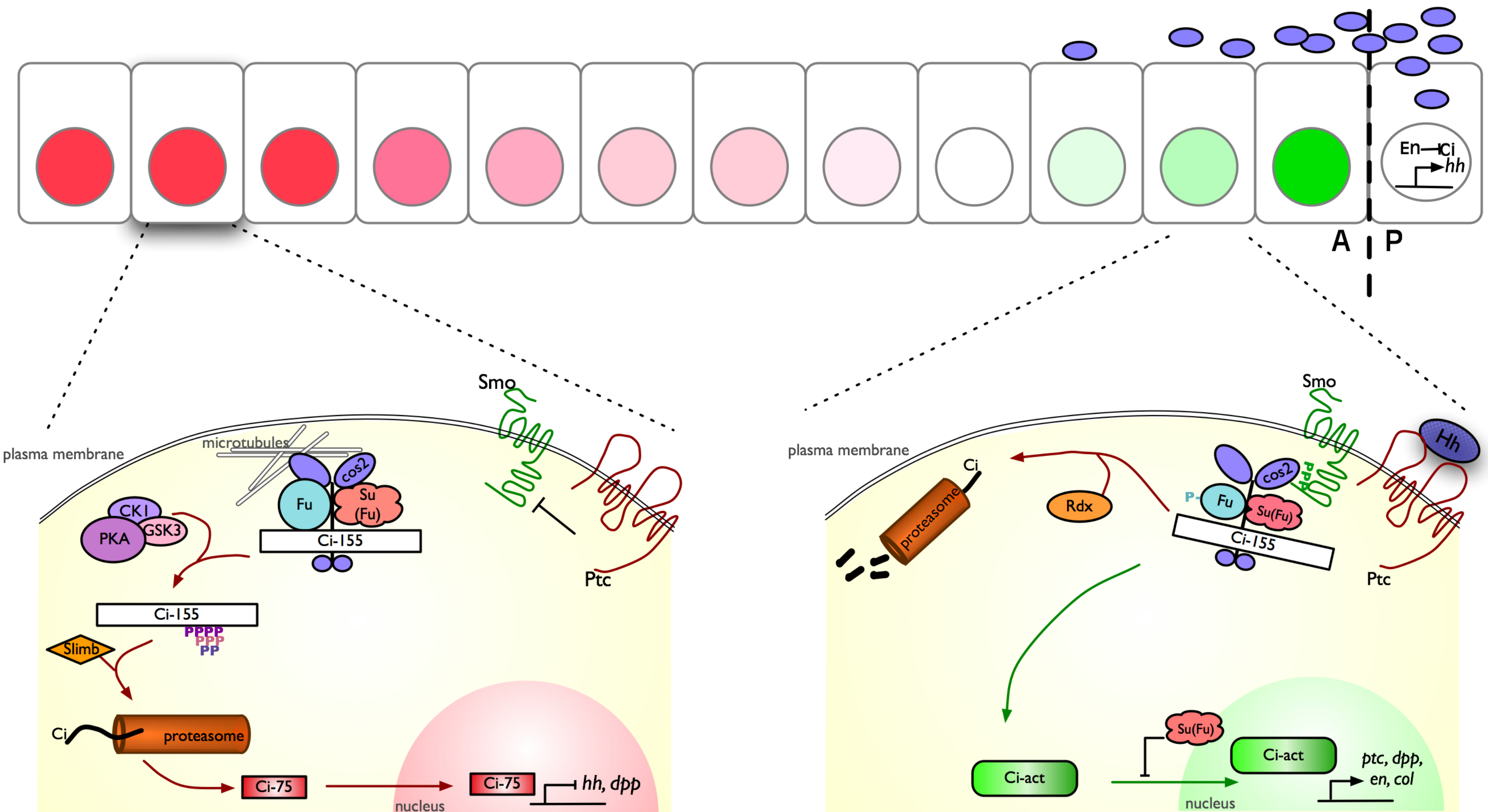


Figure 2: Diagram of Hedgehog signaling pathway in the Drosophila wing disc. Ci is expressed throughout the anterior compartment. Hh protein is secreted by posterior cells in which Engrailed represses ci, allowing the transcription of hh (top). In the absence of Hh signaling, such as in the far anterior of the wing disc, Ci-155 is cleaved to produce the repressor form Ci-75 (left). In border cells that receive the Hh signal, Ci-75 formation is blocked and Ci-155 becomes a transcriptional activator (right).

Ptc is a twelve-pass transmembrane protein with structural similarity to transporters and channel proteins (Hooper and Scott, 1989; Nakano et al., 1989); it is believed to be the Hh receptor (Chen and Struhl, 1996; Ingham et al., 1991; Lu et al., 2006). Without Hh, Ptc suppresses the Hh signaling pathway by repressing the activity of Smoothened (Smo), a seven-pass transmembrane protein with homology to G-protein coupled receptors (Alcedo et al., 1996). In cells that do not receive the Hh signal (such as anterior cells far from the A/P border), Ptc catalytically prevents Smo from signaling (Taipale et al., 2002) to downstream effectors, such as a complex containing Ci, Fused (Fu, a ser/thr kinase), Costal-2 (Cos-2, a kinesin-like protein), and Suppressor of Fused (Su(Fu), a novel protein containing a PEST domain) (Stegman et al., 2000).

How the Hh signal is transduced from Ptc and Smo to Ci is still not well understood, although recent work on these two transmembrane proteins have answered some questions. It has been found that upon binding to Hh, Ptc removes from the cell surface and Smo is phosphorylated and stabilized (Denef et al., 2000). Ptc appears to positively regulate Ci cleavage, because cleavage is inhibited and activated Ci-155 accumulates in clones expressing a dominant-negative *ptc* allele (Johnson et al., 2000). Perturbation of lipid rafts (and presumably Ptc and/or Smo function) using lipophorin knockdowns also blocks formation of Ci-75, but does not activate Ci-155 (D. Panakova, personal communication). Finally, a direct interaction between Smo and Cos-2 may play a role in transmitting the Hh signal to Ci (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003).

In the absence of Hh signaling, a complex containing Ci, Fu, and Cos-2 is bound to microtubules (Robbins et al., 1997; Sisson et al., 1997), and Ci-155 is cleaved to form Ci-75 which translocates to the nucleus where it represses target genes such as *hh* (Aza-Blanc et al., 1997). In cells that receive the Hh signal, Cos-2 does not bind to microtubules, releasing the Ci/Fu/Cos-2 complex from the cytoskeleton. Formation of Ci-75 is blocked and levels of full-length Ci-155 are elevated at the border (Aza-Blanc et al., 1997). This Hh-dependent shift in forms of Ci at the A/P border results in the transcriptional activation of *ptc*, *dpp*, and *en* (Blair, 1992). The observations that Cos-2 interacts with Smo and microtubules (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003), *cos-2* loss of function clones cannot make Ci-75 (Sisson et al., 1997), and that Ci-75 formation requires coexpression of Fu and Cos-2 in an *in vitro* baculovirus system (Robbins et al., 1997) suggest that Cos-2 functions as a scaffolding protein for Smo/Cos/Ci/Fu/Su(fu) complex formation and is required for Ci cleavage. Fused, a serine/threonine kinase, is phosphorylated in the presence of Hh signaling (Therond et al., 1996), but the significance of this phosphorylation remains unknown. It has also been shown that Cos-2 has a Fu phosphorylation site (Nybakken et al., 2002) and that Fused phosphorylation of Cos-2 attenuates Cos-2's association with Smo (Liu et al., 2007). No other targets of Fu have been identified. Su(fu) has long been the most mysterious component of the Hh signal transduction pathway, since the discovery that a *su(fu)* loss-of-function mutant suppresses *fused* null phenotypes, yet lacks a phenotype on its own (Monnier et al., 1998). However, there is some evidence that this

novel protein plays a role in the transport of Ci between the cytoplasm and the nucleus (Méthot and Basler, 2000; Sisson et al., 2006; Wang et al., 2000b).

Ci modification involves proteins that have not yet been found in the complex discussed above. For example, Protein Kinase A (PKA) antagonizes Hh signaling by phosphorylating Ci at multiple sites (Chen et al., 1999b; Chen et al., 1998; Price and Kalderon, 1999; Wang et al., 1999). These PKA phosphorylation sites prime Ci to be subsequently phosphorylated by Shaggy/Glycogen Synthase Kinase 3 (Sgg/GSK3β) and Casein Kinase I (CKI) (Jia et al., 2002; Price and Kalderon, 2002). Mutagenesis of these sites produces a mutant Ci protein that cannot form Ci-75 and functions as a constitutive activator in the absence of Hh. It has been shown *in vitro* that Ci hyperphosphorylation by PKA/ GSK3β/CKI enhances binding of Ci to a protein implicated in targeting substrates for ubiquitination called *supernumerary limb* (*slimb*) (Jia et al., 2005; Smelkinson and Kalderon, 2006).

Slimb is another negative regulator of Hh signaling that positively regulates Ci cleavage, because cleavage is inhibited and activated Ci-155 accumulates in *slimb* null clones (Jiang and Struhl, 1998; Theodosiou et al., 1998). Slimb contains a conserved F-box/WD40-repeat and is related to Cdc4p, a yeast protein involved in targeting cell-cycle proteins for ubiquitination and degradation by the proteasome. An F-box protein is the substrate recognition component of a SCF-E3 ubiquitin ligase complex that specifically recognizes a substrate that is then ubiquitinated by the complex. It has been shown that

mutations in other components of the SCF, such as Roc1 (Noureddine et al., 2002) and Cullin-1 (Ou et al., 2002), also block Ci-75 formation. Based on these data, it has been proposed that Slimb targets phosphorylated Ci-155 to an E3 complex containing Roc1 and Cullin-1. The polyubiquitinated Ci is then targeted to the proteasome, where it is unfolded and then specifically cleaved to form Ci-75, due to the presence of a simple sequence region (SSR) and a folded domain (the DNA binding zinc fingers) (Tian et al., 2005).

While much of the research described above has focused on the production of Ci-75, recent work has also shown that regulation of full-length Ci is also crucial to proper patterning. *Roadkill* (*rdx*)/*Hh induced MATH and BTB protein* (*hib*) is a Hh target and Rdx appears to target full-length Ci for Cullin-3 ubiquitination and degradation by the proteasome (Kent et al., 2006; Zhang et al., 2006).

My work has focused on understanding how posttranslational modification of Ci affects Ci function in Hh signaling and patterning. While many conditions that disrupt Hh signaling (such as *ptc* and *slimb* null clones and Ci constructs lacking PKA phosphorylation sites) simultaneously block Ci-75 formation and activate Hh target genes, it is important to note that blocking formation of Ci-75 does not necessarily lead to production of Ci activator. For example, a Ci protein with a deletion of amino acids 611-760 does not produce Ci-75, and is not constitutively active in the absence of Hh (Méthot and Basler, 1999). This observation led to studies discussed in Chapter 2, where I used site directed mutagenesis to generate a series of Ci deletion mutants and expressed them *in vivo* in the

Drosophila wing disc. My studies identified a five amino acid motif that is required for cleavage of Ci-155 to Ci-75. Significantly, a mutant Ci protein missing this motif can activate transcription normally. This motif is found only in Gli2 and Gli3, the vertebrate Gli homologs that also undergo cleavage. It is not present in Gli1, the vertebrate homolog that is not cleaved. My findings suggest alternative models for Ci repressor formation. In Chapter 3, I discuss experiments that examined two different C-terminal regions of full-length Ci. Both are downstream of the putative CBP interaction domain. The Δ1352-1398 region has significant homology to Gli2 and Gli3, and appears to play a role in nuclear export or cytoplasmic retention, as well as being crucial for transcriptional activation. The second region, Δ1161-1351, has no apparent sequence homology and seems to be important for blocking transcriptional activation and decreasing protein stability. These studies of repressor formation and transcriptional activation of Ci further our understanding of how developmental signals such as Hh that are received at the cell surface become transcriptional readouts in the nucleus.

Chapter 2

A motif required for formation of Ci-75, the Ci repressor

Introduction

Hedgehog (Hh) signaling plays an essential role in multiple developmental processes, including patterning of the *Drosophila* embryo and imaginal discs, cell fate specification in the vertebrate neural tube, and limb morphogenesis in vertebrates (reviewed in McMahon et al., 2003). Cubitus interruptus (Ci), a zinc finger transcription factor, is the only identified direct downstream effector of Hh signaling in *Drosophila*. *ci* is one of the segment polarity genes required for correct A/P patterning of embryonic segments (Nusslein-Volhard and Wieschaus, 1980) as well as patterning of the larval imaginal discs (Domínguez et al., 1996). The vertebrate homologs of *ci*, the *gli* genes, are similarly important in vertebrate patterning and have been implicated in processes such as floor plate differentiation (Ding et al., 1998), digit formation (Kang et al., 1997), and facial development (Hui et al., 1994).

Increasing evidence suggests that developmental signaling pathways in general and Hh signaling in particular, play crucial roles in tumorigenesis. The *gli* genes were first identified as genes amplified in a human glioma cell line (Ruppert et al., 1988). The importance of the role of *glis* in tumorigenesis has been further substantiated in studies of various epithelial, digestive tract, and brain cancers (reviewed in Kasper et al., 2006).

Furthering our understanding of how Hh regulates Ci function will therefore provide insight into how Ci activity contributes to both development and metastasis.

One of the earliest responses to Hh signaling is the suppression of Ci cleavage to form a transcriptional repressor. Although full-length *ci* mRNA is uniformly expressed throughout the anterior (A) compartments of both *Drosophila* embryos and imaginal discs and is translated as a 155kD polypeptide (Ci-155) (Schwartz et al., 1995), full-length Ci protein is proteolytically cleaved to form a smaller, 75kD transcriptional repressor (Aza-Blanc et al., 1997) in the absence of Hh signaling. Ci protein primarily exists in this shorter repressor form, and Ci activator (Ci-act) is only present near the A/P border where Hh signaling is active.

There have been several reports of Hh activity in situations in which Ci protein levels have been reduced (Gallet et al., 2000; Lewis et al., 1999; Suzuki and Saigo, 2000), as well as Hh targets that lack Ci/Gli binding sites (Krishnan et al., 1997a; Krishnan et al., 1997b; Lessing and Nusse, 1998). Taken together, these studies would seem to suggest the existence of alternative Hh signaling pathways that work through other transcription factors that are not Ci. However, genetic analysis in *Drosophila* demonstrated that these examples of Ci-independent Hh signaling may actually be due to derepression of Ci-75 targets (Méthot and Basler, 2001), suggesting that Ci-75 activity plays a more extensive role in Hh signaling than previously believed.

Despite the fact that Ci-75 plays a crucial role in Hh signaling, very little is known about the process of Ci-75 formation and what little is known is rather controversial. It has been proposed that Supernumerary limb (Slimb) specifically recognizes hyperphosphorylated Ci and targets it for ubiquitination and subsequent cleavage by the proteasome (Jiang and Struhl, 1998; Theodosiou et al., 1998). However, Ci lacks a canonical Slimb binding site, and no one has detected polyubiquitinated forms of Ci when proteasome function is blocked. Although the vast majority of the proteins targeted to the proteasome are unfolded into polypeptides and systematically degraded (reviewed in Ciechanover and Schwartz, 1998), it has been proposed that Ci contains a bipartite signal consisting of a tightly folded domain followed by a simple sequence region (SSR) that allows for the release of Ci-75 before degradation can be completed (Tian et al., 2005). Proteasome-mediated cleavage that generates a precisely defined and active product is not without precedent (it has been demonstrated in NF-κB (Palombella et al., 1994)), but it is not clear how such a mechanism might apply to Ci.

This chapter describes the identification and study of a *cis* element in Ci that is required for the formation of Ci-75. I used site directed mutagenesis to generate a series of transgenic Ci constructs that I characterized using an *in vivo* wing disc assay. I discovered that deletion of the *cis* element blocks proteolytic conversion of a tagged Ci-155 to Ci-75. Such deletion mutants do not lead to Ci-act formation in the absence of Hh signaling. Interestingly, this *cis* region is downstream of both the cleavage site and the SSR; my data indicates that it contains a motif required for Ci-75 formation rather than the cleavage site

itself. A stretch of residues in this region appears to be conserved in several of the Gli proteins, the mammalian homologs of Ci, suggesting that both Ci and the Glis undergo cleavage in a conserved manner. Finally, I present data indicating that the SSR is not necessary for Ci-75 formation.

Results

Ci is translated as a single polypeptide chain containing 1,397 amino acids (Figure 1). The DNA binding domain contains five zinc fingers, and is located at amino acids 453-603 (Pavletich and Pabo, 1993). The only identified activation domain (CBP AD) is the CREB Binding Protein (CBP) interaction region that is located near the C-terminal end of the protein at residues 1020-1160 (Akimaru et al., 1997). Ci-75 behaves as a repressor presumably because cleavage removes the CBP AD.

To study Ci cleavage, an artificial 76 kD Ci construct (Ci-76) truncated at amino acid 703 was expressed in Schneider S2 cells (a *Drosophila* embryo derived cell line that does not express Ci endogenously) (Aza-Blanc et al., 1997). Because Ci-76 runs at a higher apparent molecular weight on a SDS-PAGE gel than endogenous Ci-75 produced in imaginal disc derived clone-8 (Cl-8) cells, it was previously estimated that the cleavage site to generate Ci-75 lies between the zinc finger domain and amino acid 703, or between amino acid residues Leu-650 and Asp-700. One caveat of this approximation is the observation that the molecular weight of Ci appears to vary significantly depending on the source of the sample (see Figure 2A in Aza-Blanc et al., 1997). A conflicting report suggested that cleavage occurs in the 713-760 region because deletion of residues 611-760 blocks cleavage whereas deletion of residues 611-712 had no effect (Méthot and Basler, 1999).

All other reported mutations of Ci that block Ci cleavage also generate Ci-act independent of Hh signaling. For example, there are PKA phosphorylation sites in Ci, which serve as recognition sites for Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3β (GSK3β), that simultaneously promote Ci-75 formation and suppress Ci activation when phosphorylated (Jia et al., 2002; Jia et al., 2005; Price and Kalderon, 1999). Another mutation that blocks Ci repressor formation and generates Ci-act independent of Hh signaling is a deletion of the first two zinc fingers in Ci (Croker et al., 2006). It is believed that these mutations simultaneously block repressor formation and promote activation because these domains normally function to both direct Ci to a repressor formation complex and simultaneously prevent association with a Ci activation complex.

An *in vivo* wing disc assay for Ci-75 formation. In order to identify the amino acid residues in Ci that are required to generate Ci-75, a series of tagged transgenic constructs (Figure 1B) were synthesized and expressed using the *Drosophila* wing imaginal disc as an *in vivo* assay. All constructs have a N-terminal 3X HA epitope tag and a C-terminal 3X Myc epitope tag in order to facilitate detection of transgenic protein in a wild-type background. Attempts to develop a cell culture assay for Ci-75 formation in either S2 cells or Cl8 cells were unsuccessful due to the prevalence of multiple nonspecific cleavage products (data not shown). The primary advantages of using wing discs are that all components of the Hh signaling pathway are intact, background noise is significantly less than observed with cultured cells, and the *UAS-GAL4* system allows controlled spatial

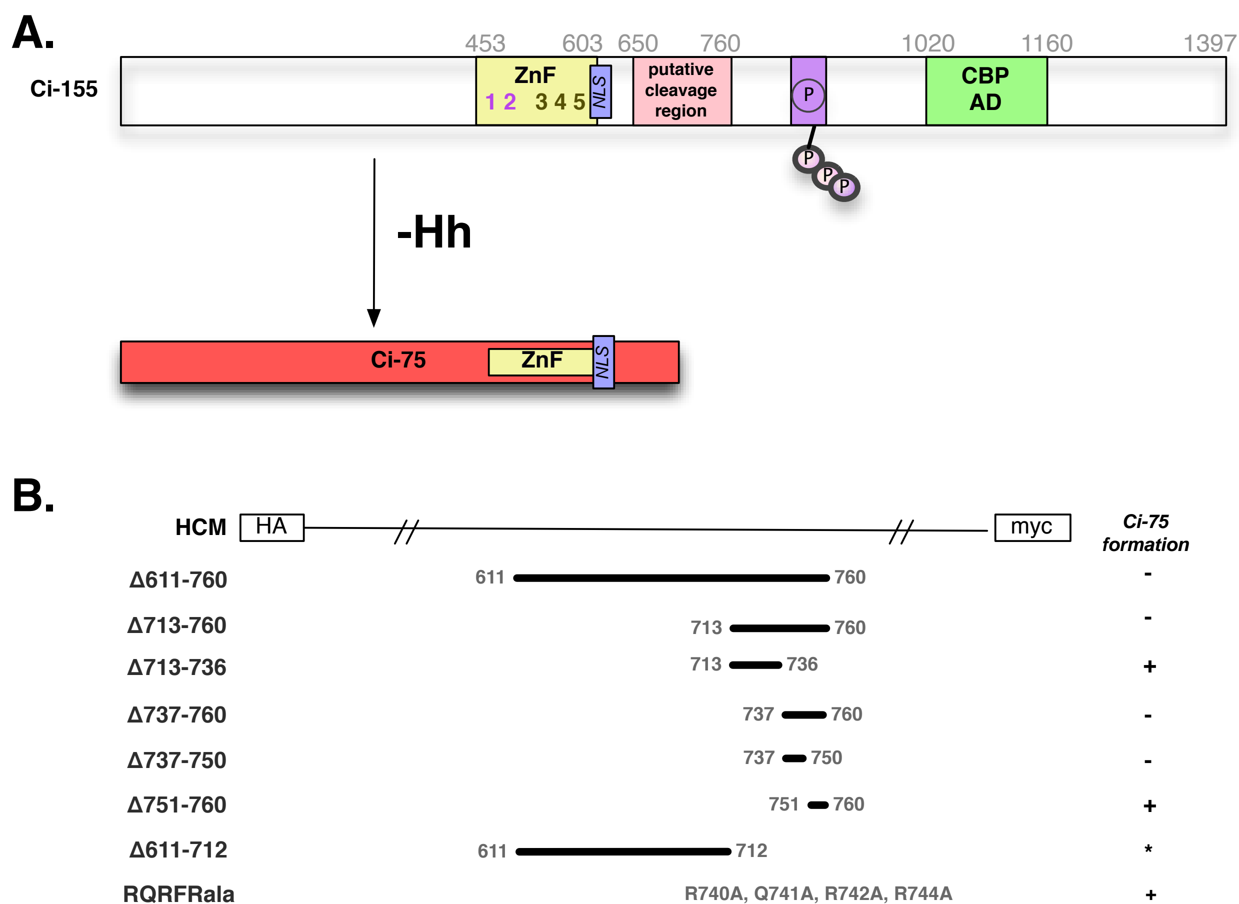


Figure 1: Schematic representations of Ci and transgenic Ci constructs. (A) Full length Ci-155 and cleavage product Ci-75. N-terminus is to the left, C-terminus to the right. Shown are the zinc finger domain (ZnF) with individually numbered zinc fingers 1-5, nuclear localization signals (NLS), a putative cleavage region, a regulatory region (P) containing PKA/GSK3β/CK1 recognition sites, and CBP binding activation domain (CBP AD). In the absence of Hh signaling, Ci-155 is cleaved to produce the repressor Ci-75. (B) Table of tagged transgenic constructs with black bar indicating deleted residues and effect on Ci-75 formation.

expression of transgenic constructs in either Hh responding or non-responding cells (Brand and Perrimon, 1993).

Expression of a HA- and Myc- tagged wild-type Ci construct (HCM) using the *C765-GAL4* driver, which drives expression uniformly throughout the wing disc, produces an active, full-length protein that can be cleaved to produce a HA-Ci-75 form, as detected using an anti-HA immunoblot of wing disc lysates (Figure 2A). The protein band corresponding to HA-Ci-75 is not present when *UAS-HCM* is driven using the *engrailed-GAL4* (*en-GAL4*) driver, which expresses only in the posterior of the wing disc where Hh is produced and Ci is normally absent. Thus, the presence of HA-Ci-75 is Hh dependent because the Hh in the posterior compartment prevents HCM from being processed to HA-Ci-75 in P cells. Expression of HCM has no effect in the anterior compartment, but has a phenotypic effect in the posterior compartment in the adult wing blade compared to wild type (Figure 2B). The HCM construct therefore produces functional Ci protein as evidenced by the ectopic vein formation, blistering, and overgrowth in the posterior compartment (Figure 2B). The anterior compartment is relatively normal, because HCM is cleaved to make HA-Ci-75 and does not act as a transcriptional activator in the absence of Hh.

Determination of Ci region required for repressor formation. It has been previously reported that deleting amino acid residues 611-760 blocks Ci-75 formation (Méthot and Basler, 1999). I confirmed this observation by generating a HA- and Myc-tagged Ci construct in which these residues were deleted (Δ611-760). *UAS-Δ611-760*

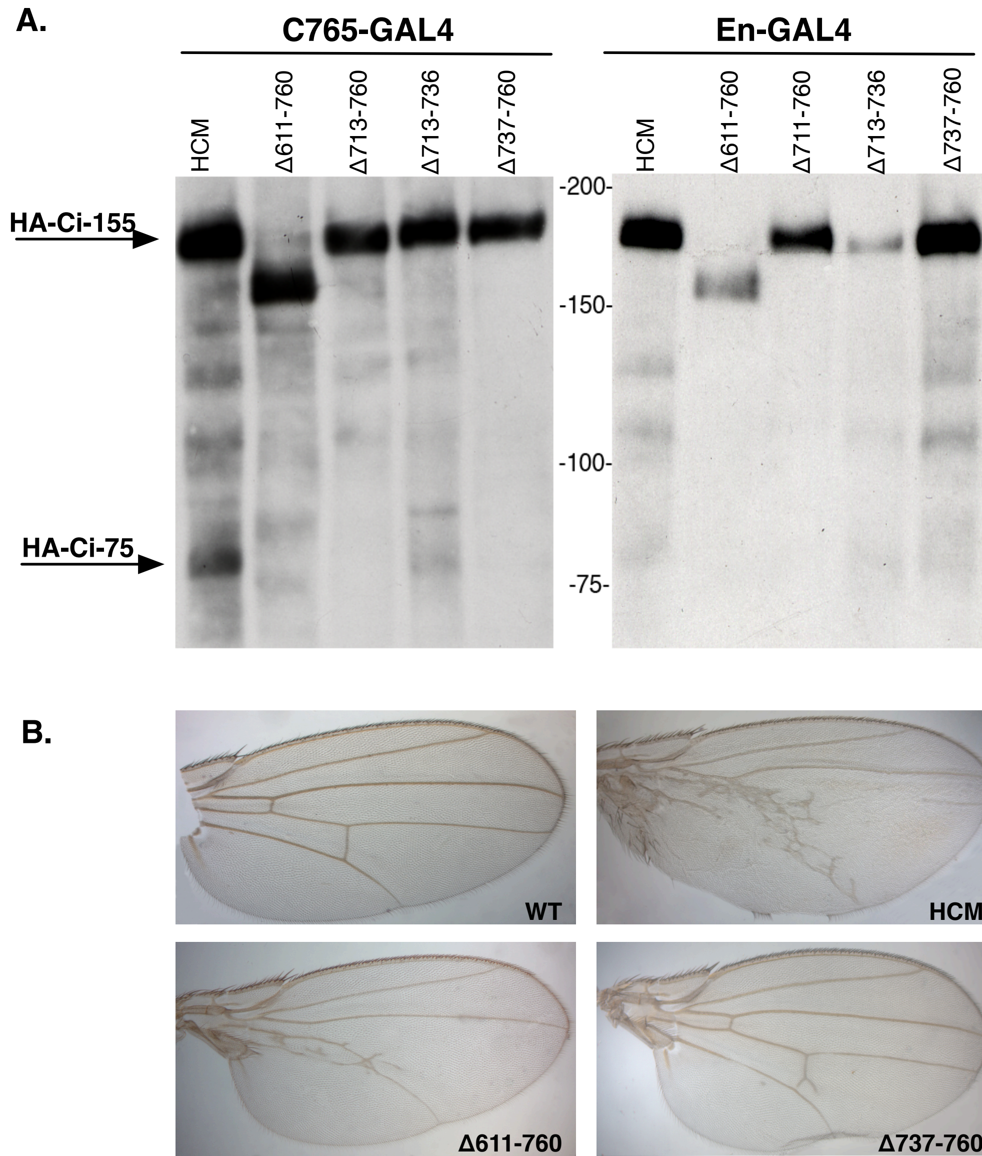


Figure 2: Residues 737-760 are required for Ci-75 formation and activation of Ci constructs is Hh dependent (A) Anti-HA Western blot of wing disc lysates in which HA-tagged constructs were expressed throughout the wing disc using the *C765-GAL4* driver (*left*) or the posterior *en-GAL4* driver (*right).* (B) Adult wing phenotypes of HA-tagged Ci constructs expressed throughout the wing disc using the *C765-GAL4* driver.

produces a protein that cannot be cleaved to produce a HA-Ci-75 form, as detected by an anti-HA immunoblot of wing disc lysates (Figure 2A). Several other Ci proteins with different subsets of residues deleted (Δ713-760, Δ713-736, and Δ737-760) were also constructed and analyzed in order to identify which residues between 611-760 are required for Ci-75 formation. Using the *C765-GAL4* driver, a band corresponding to HA-Ci-75 is observed in wing discs expressing *UAS-Δ713-736* but not *UAS-Δ611-760* or *UAS-Δ737-760*. These results suggest that only residues 737-760 are necessary for Ci-75 formation and that residues 611-736 are not (Figure 2A).

Blocking Ci-75 formation is not sufficient to activate target gene expression. Deletions in the 611-760 region do not affect the mutant Ci protein's ability to activate target genes when exposed to Hh signal, indicating that these proteins are functional. For example, Δ713-760 and Δ737-760 Ci proteins have posterior phenotypes with ectopic overgrowth similar to those of HCM (Figure 2B). Blocking Ci-75 formation in Δ713-760 and Δ737-760 does not lead to overgrowth in the anterior, supporting previous observations that Hh signaling is not simply a result of relieving Ci-75 repression of target genes and that Hh signaling requires some form of Ci-act (Aza-Blanc et al., 1997; Méthot and Basler, 1999). Although Δ713-760 and Δ737-760 do not make HA-Ci-75, blocking repressor formation is not sufficient to activate transcription and Δ713-760 and Δ737-760, like HCM, require Hh signaling to be further transformed into a Ci-act form, the precise nature of which is still unknown.

Residues 737-760 contain a motif required for cleavage that is distinct from the actual cleavage site. The determination that residues 737-760 are required for cleavage was unexpected given that the site of cleavage to produce Ci-75 had been estimated to be between residues 650-700 (Aza-Blanc et al., 1997). My attempts to unequivocally identify the cleavage site by purifying either Ci-75 or the C-terminal cleavage product for mass spectroscopy using immunoprecipitation and a variety of antibodies to HA, Myc, and to Ci itself have proven unsuccessful.

In order to obtain a more precise estimate of the cleavage site without using mass spectroscopy, a HA tagged Ci construct truncated at amino acid residue 760, *UAS-HACiΔ760*, was utilized. If Ci is cleaved in the 737-760 region, the HA-Ci-75 band from wing disc lysates expressing HCM driven by *C765-GAL4* would be approximately the same size as HA-Ci-Δ760. However, as shown in Figure 3A, HA-Ci-75 is 12 kD smaller than HA-Ci-Δ760, indicating that residues 737-760 contain sequences required for cleavage that are downstream of the cleavage site (illustrated in Figure 3B). Based on the peptide sequence of Ci, it is estimated that the terminal residue of a HA-Ci-75 protein that is 12 kD smaller than HA-Ci-Δ760 would be located around residue 645. This estimate is consistent with previous work.

Interestingly, the HA-Ci-75 band is significantly fainter than the HA-Ci-Δ760 band despite the fact that full length HCM is expressed at significantly higher levels than

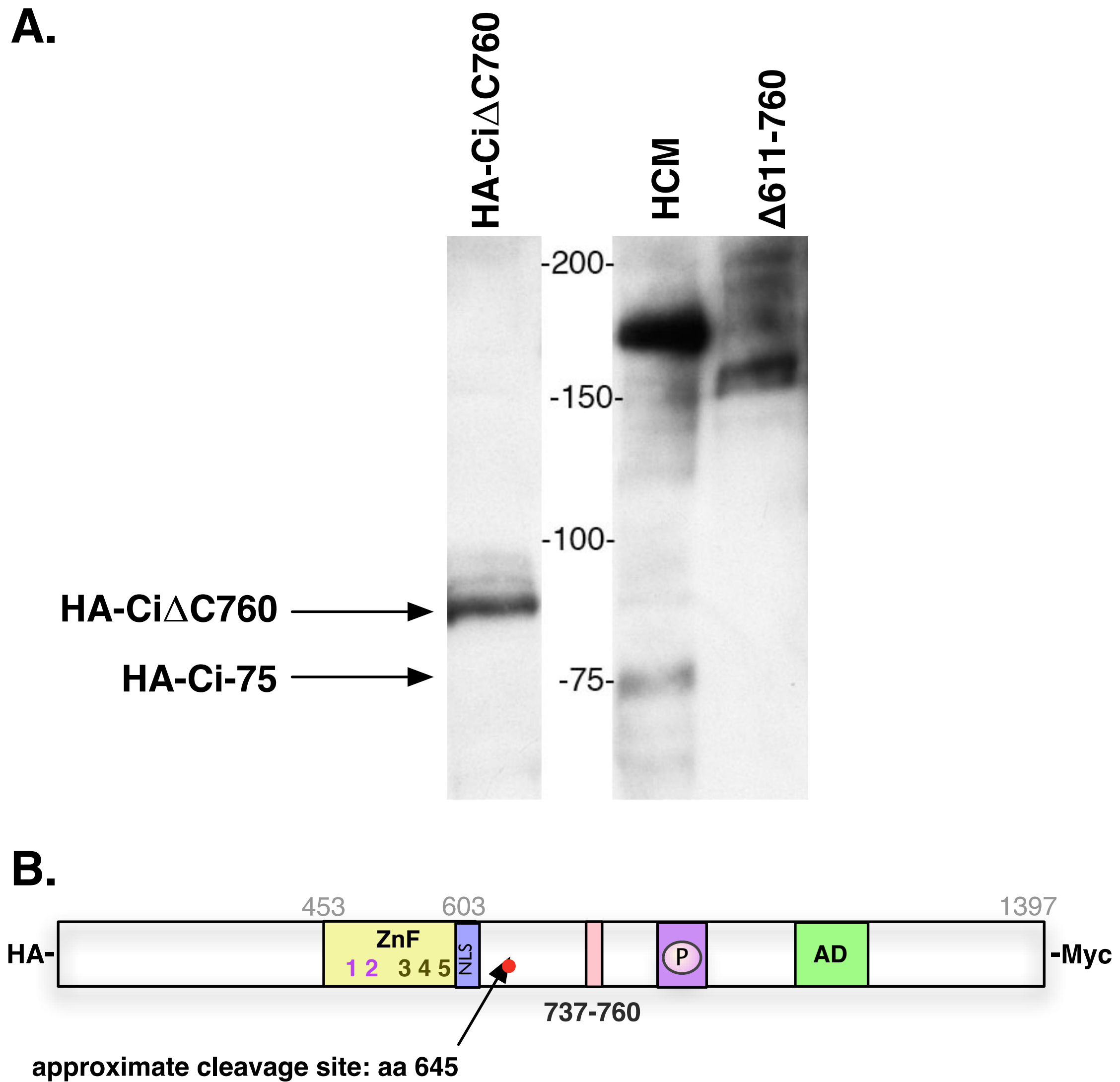


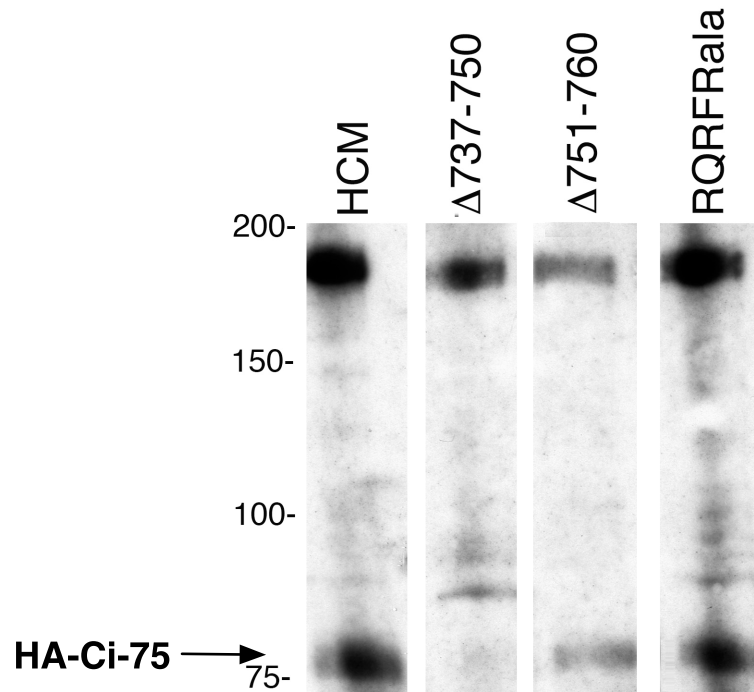
Figure 3: HA-CiΔ760 migrates as a larger protein compared to HA-Ci-75. (A) Wing disc lysates expressing *C765-GAL4* driven HA-tagged Ci constructs as indicated were separated on a 7.5% SDS-PAGE gel and immunoblotted with anti-HA antibody. HA-CiΔ760 (top arrow) is approximately 12 kD larger than HA-Ci75 (bottom arrow). Δ611-760 was included as a control for antibody background. (B) Schematic representation of Ci with red bullet (•) indicating putative location of cleavage site relative to motif required for Ci-75 formation.

HA-Ci-Δ760. This observation suggests either that rates of HA-Ci-75 formation are low or that HA-Ci-75 is unstable.

Residues 737-750 are required for cleavage and are rich in charged and polar amino acids. To further refine the location of the Ci cleavage motif, two other transgenic Ci constructs were generated that deleted either residues 737-750 or 751-760. Using the *C765-GAL4* driver, a band corresponding to HA-Ci-75 is observed in wing discs expressing *UAS-Δ751-760* but not *UAS-Δ737-750*, indicating that residues 737-750 are required for Ci-75 formation (Figure 4A).

While the 737-750 residues are required for Ci-75 formation, they are not sufficient for Ci cleavage. No HA-Ci-75 band was present when HA-Ci-Δ760, which contains residues 737-750, is expressed throughout the wing disc (Figure 3A). Therefore, additional Ci sequences or structural motifs downstream of residue 760 must also be required for Ci cleavage.

BLAST searches and CLUSTALW alignments of either the entire Ci sequence or region 737-750 did not identify any residues in 737-750 that are conserved in any of the Gli proteins (data not shown). However, a visual inspection of the thirteen amino acids in 737-750 (Figure 4B) reveals several positively charged residues (arginine, lysine) and polar residues (glutamine). To determine if these residues are required, site directed mutagenesis of HCM was used to mutate amino acids in the first half of 737-750 (R740, Q741, R742,



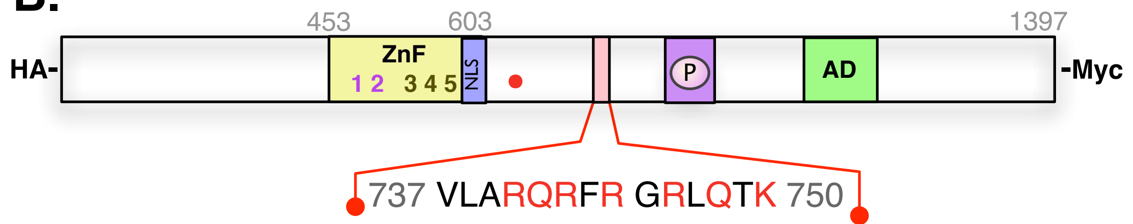


Figure 4: Region 737-750 is required for Ci-75 formation and is rich in positively charged or polar residues. (A) Wing disc lysates expressing *C765-GAL4* driven HA-tagged Ci constructs as indicated were separated on a 7.5% SDS-PAGE gel and immunoblotted with anti-HA antibody. (B) Ci schematic indicating amino acid sequence of region 737-760. Polar and charged residues are in red.

and R744) to alanine in order to create the transgenic construct *UAS-RQRFRala*. As Figure 4A shows, HA-Ci-75 is observed in lysates of wing discs expressing *UAS-RQRFRala* driven by *C765-GAL4,* indicating that these residues are not required for Ci cleavage.

T-COFFEE sequence alignments uncover a similar conserved motif in Gli2 and Gli3. In vertebrates, the activator and repressor functions of Ci have been subdivided amongst Gli1, Gli2, and Gli3, the three vertebrate homologs of Ci. Gli3 exists primarily as a processed transcriptional repressor (Wang et al., 2000a); Gli2 has both activator and repressor activity (Pan et al., 2006); and Gli1 is not cleaved and has only transcriptional activator activity (Dai et al., 1999; von Mering and Basler, 1999). If the formation of cleaved Ci/Gli repressor were conserved between vertebrates and *Drosophila*, it would seem likely that at least Gli2 and Gli3 would have a region homologous to Ci residues 737-750. However, as indicated above, BLAST searches and CLUSTALW alignments of Ci and the Gli proteins did not reveal any significant homologies outside of the zinc finger domains.

One caveat of this negative result is that these types of alignments do not identify very short stretches of homology. For example, PKA recognition sites common to Ci, Gli2, and Gli3 are not recognized. Therefore, a slightly modified approach was taken, in which very short regions were compared to consensus sequences. Consensus Gli1, Gli2, Gli3 sequences were compiled from rat, mouse, chicken, zebrafish, pufferfish, and human sequences using T-COFFEE (Notredame et al., 2000), based on the reasoning that motifs important for protein function should be conserved across species. These consensus

sequences were then aligned using T-COFFEE with the amino acid sequence GRLQTK (Ci residues 745-750), since R740, Q741, R742, and R744 are not required for Ci-75 formation (see Figure 4). The results of this analysis revealed a very similar sequence (RLKAK) that is present in both Gli2 and Gli3 proteins (Figure 5). A less conserved sequence (MLKAK) was also found in the Gli1 protein (Figure 5). The conservation of this RLQTK motif in Ci and the Gli proteins is consistent with the hypothesis that this is a region required for Ci-75 cleavage.

Not all deletions in region Δ611-760 block Ci cleavage. The observation that some deletions in the 611-760 region generated Ci-75 further supports the validity of the wing disc as an *in vivo* system to assay Ci-75 formation. Deletion constructs on either side of the RLQTK motif, Δ713-737 shown in Figure 2A and Δ751-760 shown in Figure 4A, do not block repressor formation when expressed using *C765-GAL4*. However, there have been conflicting reports as to whether residues 611-712 are required for Ci-75 formation.

It has been proposed that the proteasome specifically cleaves Ci-155 to form Ci-75 and that the Ci signal for cleavage is bipartite, consisting of (1) a folded domain (ZnF) and (2) a region of amino acids with low complexity known as a Simple Sequence Region (SSR) (Tian et al., 2005). These authors identified residues 611-712 as containing the SSR in Ci and cited the observation that deletion of 611-760 blocks Ci-75 formation in support of

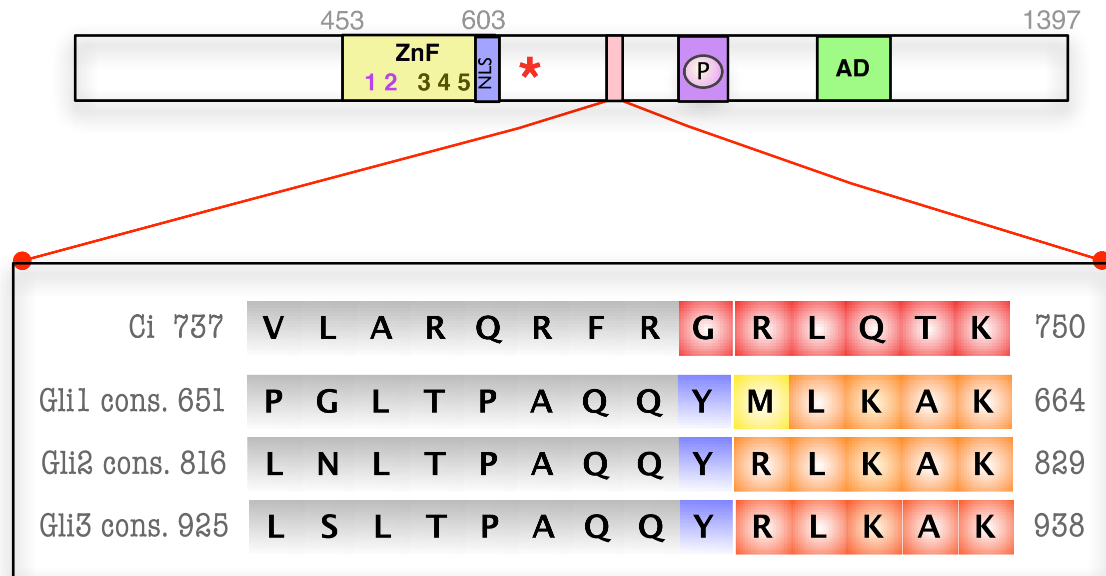


Figure 5: T-COFFEE analysis of Ci sequence and Gli consensus sequences. Amino acid sequence GRLQTK from *Drosophila melanogaster* Ci protein sequence was aligned with consensus sequences of vertebrate Gli proteins using T-COFFEE. Intensity of red color is measure of similarity to Ci, with blue indicating no similarity and red indicating high similarity.

their model. Contradicting this result, the Basler lab has reported that residues 611-712 are not required to form Ci-75 (Méthot and Basler, 1999).

A *UAS-Δ611-712* construct was generated to resolve this discrepancy. Expression of *UAS-Δ611-712* using the *C765-GAL4* driver in wing discs produces a Ci cleavage product, HA-Ci-67*, that is approximately 8 kD smaller than HA-Ci-75 (Figure 6). The presence of this Ci cleavage product derived from Δ611-712 argues either that the 611-712 region is not the SSR or that the bipartite proteasome specific cleavage model for Ci-74 formation needs revision. The observation that Δ611-712 produces a HA-Ci-75-like cleavage product that is smaller than HA-Ci-75 may provide insight into the mechanism of Ci-75 formation.

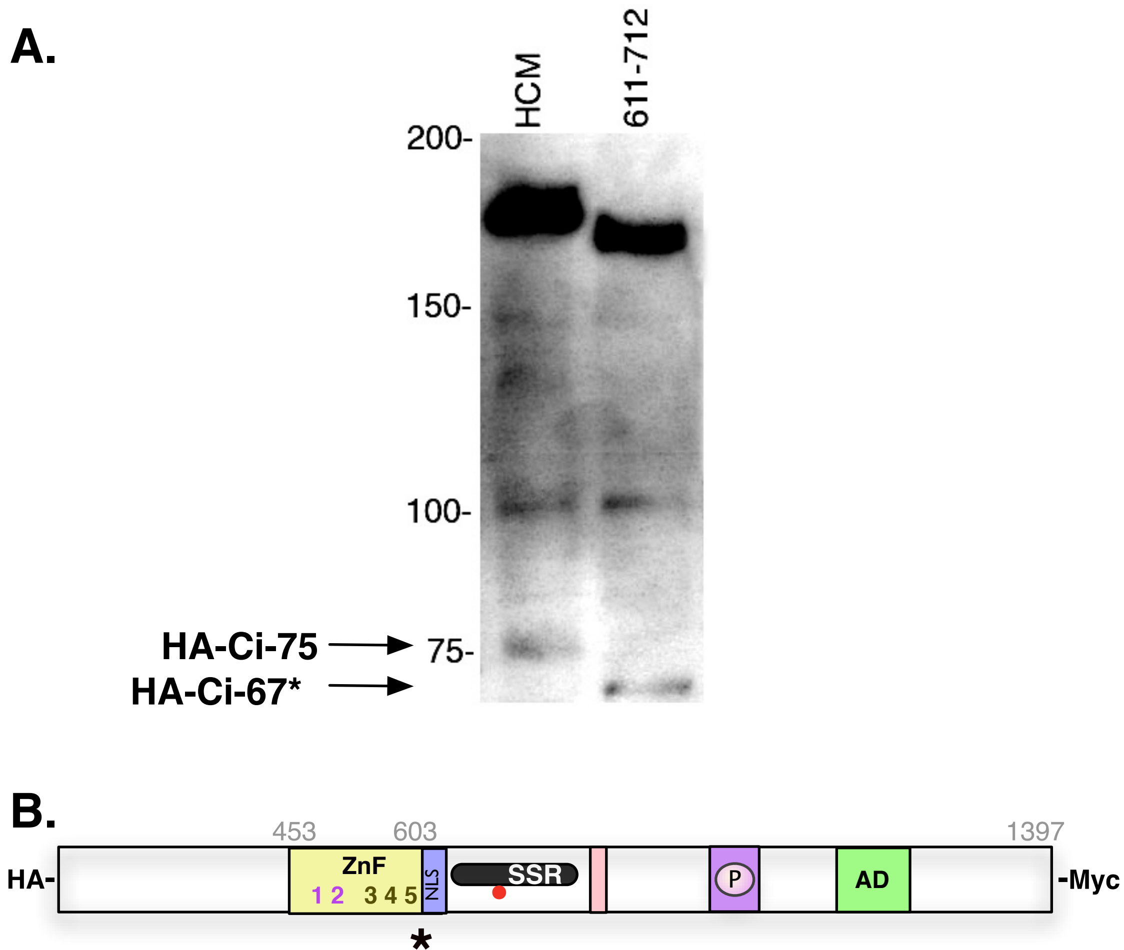


Figure 6: Deletion of Simple Sequence Region (SSR) does not block formation of Ci-75. (A) Wing disc lysates expressing HA-tagged Ci constructs as indicated were separated on a 7.5% SDS-PAGE gel and immunoblotted with anti-HA antibody. HA-Ci-67* indicates a smaller form of Ci that is generated in wing discs expressing Δ611-712. (B) Schematic representation of Ci with SSR 611-712 indicated as a black bar. Approximate site of cleavage to form HA-Ci-67* (asterisk) as compared to approximate site of cleavage to generate HA-Ci-75 (red bullet).

Discussion

In this study, I have identified a sequence required for Ci repressor formation. This sequence is the fourteen amino acid residues from Ci-737 to Ci-750, here after referred to as the Repressor Formation Domain (RFD). The RFD is rich in positively charged and polar residues and contains a five amino acid motif (RLQTK) that appears to be conserved in the vertebrate Gli proteins that also undergo cleavage, Gli2 and Gli3. I have shown that the RFD is distinct from the cleavage site and located approximately 12kD downstream. Finally, I have demonstrated that deletion of the SSR of Ci does not block repressor formation, although the cleaved protein is significantly smaller than Ci-75.

Using the wing disc to assay Ci-75 formation rather than S2 cells or an *in vitro* system had several advantages. Because many cell lines, such as S2 cells, do not express some components that are necessary for Hh signaling, completely recapitulating Hh signaling in these cells is not possible. Given the observation that many of the Hh signaling components involved in Ci-75 formation have specific requirements with regard to subcellular localization (i.e. Smo/Ptc trafficking at the membrane and in vesicles and Cos association with microtubules), development of an *in vitro* assay would seem problematic at best. In addition, the presence of nonspecific, Hh independent cleavage products was significantly lower in wing disc lysates compared to S2 cell lysates. The background levels in the wing disc assay were not negligible, although similar background issues can be detected in other systems that have been used to assay Ci-75 formation (Méthot and Basler, 2001; Tian et al.,

2005). With careful comparison of background band intensities in the expected molecular range of a sample to both positive and negative controls, the wing disc can be used to study the process of Ci-75 formation.

When Ci-75 was first identified as a cleavage product of Ci-155, it was reported that the amount of Ci-75 was roughly equivalent to the amount of Ci-155 in wing discs (Aza-Blanc et al., 1997). However, I have observed that the ratio of Ci-75 to Ci-155 to be consistently much lower. One possible explanation could be that detection of the N-terminal tag is less efficient compared to using AbN (the rabbit anti-N-terminal Ci antibody that was used previously), because either the anti-HA antibody is inefficient or the tag is incompatible with wing disc cells. The fact that both full-length HCM and the truncated HA-CiΔC760, are easily detectable compared to HA-Ci-75 argues against this explanation. This observation also carries across to other systems: an approach to purify HA-Ci-75 (in order to identify the cleavage site using electrospray mass spectroscopy) from *Drosophila* embryos proved unsuccessful due to low levels of HA-Ci-75 production. While it is possible that modifying Ci with multiple tags has inhibited either its ability to be processed, its targeting to the processing complex, or has increased its rate of turnover relative to untagged endogenous Ci, other labs have also reported problems detecting untagged Ci-75 using antibodies to various N-terminal Ci domains (C. Zhang, S. Ogden, personal communications). It is also possible that recovery of Ci-75 is lower relative to Ci-155 with the methods used. Nevertheless, it may be that Ci-75 production is relatively inefficient and

that other mechanisms must exist in the anterior compartment to prevent full-length Ci from activating transcription in the absence of Hh signaling.

The Ci sequence RLQTK is most conserved in the two Gli proteins, Gli2 and Gli3, which are cleaved in vertebrate systems. It remains to be seen whether deletion of the RLKAK motif in these proteins blocks cleavage in a similar manner to Δ737-750 for Ci. Such a result would strongly support the model that this motif is necessary for repressor formation. It is possible that replacing the Gli1 sequence, MLKAK, with either RLQTK or RLKAK would allow Gli1 to make repressor. Although this can and should be tested, Gli1 lacks much of the sequence homology shared by Gli2 and Gli3, and therefore may lack other domains which are required for cleavage. As I have shown for Ci, the RLQTK motif is not sufficient for Ci cleavage: no HA-Ci-75 is produced by HA-CiΔC760.

One remaining question is: "How does the RFD act to promote formation of Ci - 75?" One possibility is that posttranslational modification of the RFD in the absence of Hh signaling targets the Ci protein for cleavage into Ci-75, presumably through a protein-protein interaction with a component of a complex involved in Ci-75 formation. However, the RFD is the only motif in Ci that has been identified to date that specifically blocks Ci-75 formation without affecting Ci activation. Mutation of all other motifs, such as the PKA sites, the first two zinc fingers, and the C-terminal domains discussed in Chapter 3, that block Ci cleavage simultaneously promote Ci activation, possibly because the mutant Ci proteins are redirected from a repressor formation complex to an activation complex. If this

is the case, the RFD may not function to direct Ci into a complex for Ci cleavage in a similar manner since mutation of the RFD does not promote Ci activation.

The observation that deleting residues 611-712 produces a fragment that is 8kD smaller than HA-Ci-75 (HA-Ci-67*), suggests alternative models for Ci repressor formation. My results support a model in which the RFD functions as a recognition site for an exo-site protease, which is a type of protease that cleaves a protein at a site distinct from its recognition site (reviewed in Krishnaswamy, 2005). Alternatively, the RFD, rather than the SSR, may be required for Ci-75 formation by the proteasome. According to this model, full-length Ci-155 is unfolded and the polypeptide chain in inserted C-terminal end first into the proteasome. Subsequently the proteasome degrades the polypeptide chain until it reaches the RFD, at which point it pauses and releases a Ci protein that has been truncated approximately 8kD upstream of the RFD. In either case, one could test the hypothesis that the RFD is a proteolytic recognition site that acts as a molecular ruler to catalyze Ci cleavage at a separate site, either by varying the length of the sequence between the RFD and the cleavage site or by moving the RFD itself to another location in Ci.

Materials and Methods

Molecular biology: The plasmid *pBSKS-Ci-GFP* was generated by adding a 3X-HA epitope tag to the N terminus and a 3X-Myc epitope tag to the C terminus of a *ci* cDNA clone in *pBSKS-KCiD* (gift from P. Aza-Blanc) Triple glycine linkers were also added between each tag and the *ci* cDNA. *pUAST-GFP* and all epitope tags were gifts of D. Casso. All Ci transgenes were derived from the *pBSKS-HCM* construct and include an N-terminal 3X-HA tag and a C-terminal 3X-Myc tag. They were all cloned as KpnI-XbaI fragments into pUAST (Brand and Perrimon, 1993) and integrated into the fly genome by germ line transformation.

For RQRFRala, amino acids R740, Q741, R742, R744 were mutated into alanine residues using the Quikchange site directed mutagenesis protocol from Stratagene. The deletion constructs are named according to the residues deleted. For example, Δ611-760 lacks amino acids 611-760. All deletion mutants (Δ611-760, Δ713-760, Δ713-736, Δ737-760, Δ737-750, Δ751-760, and Δ611-760) were generated using the Quikchange mutagenesis kit (Stratagene) with the following modifications to the protocol as described below. 5'phosphorylated primers flanking the region to be deleted were synthesized commercially (GibcoBRL, Operon, Elimbio) such that the forward primer was downstream of the last amino acid to be deleted and the reverse primer was upstream of the first amino acid to be deleted. After eighteen cycles of DNA synthesis using PFUTurbo (Stratagene), the parent vector was digested using DpnI, the synthesized DNA was ethanol precipitated,

resuspended in DNA ligation buffer, ligated for 5 minutes at room temperature using a Rapid Ligation Kit (Roche), and transformed into XL1-Blue supercompetent cells. (Stratagene). Transformants were screened using DNA sequencing and positive clones were sequenced in their entirety to confirm that no second site mutations had been introduced.

Fly strains: *C765-GAL4* and *en-GAL4* were obtained from the Bloomington Fly Center.

Experimental fly crosses: *UAS-Ci* constructs were crossed to either *C765-Gal4* or *En-Gal4* drivers as described (Brand and Perrimon, 1993). Progeny from these crosses were raised at room temperature.

Adult wing blade mounts: Wings were removed from adult animals, dehydrated in isopropanol, and mounted in Euparal.

Immunoblotting: The detection of the Ci cleavage product was essentially performed as described (Aza-Blanc et al., 1997). Wandering third instar larvae were collected, rinsed in PBS, cut in half, and everted. Wing imaginal discs were transferred to PBS containing protease inhibitors (Complete; Roche) at 4°C. Discs were spun down in a microcentrifuge at 3000 rpm for 5 minutes at 4°C and resuspended in RIPA lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 10mM sodium phosphate, pH 7.2) with 0.005% DNAse on ice for an additional 5 minutes. Lysates were heated to 95°C for 5 minutes with SDS-PAGE sample buffer added.

Wing disc lysates (20-40 wing discs/lane) were separated on a 7.5% polyacrylamide gel by SDS-PAGE under reducing conditions and transferred to PVDF membranes using semidry blotting (1 hour at 1mA/cm^2). Membranes were blocked with 5% dry powdered milk in TBST (TBS + 0.1% Tween-20) overnight at 4°C. Membranes were then incubated with mouse anti-HA (1:500; Santa Cruz Biotechnology) or rat anti-HA (1:500; Roche) antibodies for one hour. Membranes were washed 4X for 15 minutes and then incubated with peroxidase-labeled secondary antibody (1:4000) (Jackson Immunoresearch) for 45 minutes. All membranes were visualized using ECL Plus Western Blotting Immunodetection System (GE Healthcare) and exposure to Kodak Biomax Light Film (Kodak).

T-COFFEE sequence alignment: Sequences to be aligned were uploaded in fasta format to the T-COFFEE server (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi). The advanced T-COFFEE settings used were Lalign_pair (10 best local alignments) and slow_pair: (accurate global alignment) as described previously (Notredame et al., 2000; Wallace et al., 2006).

Chapter 3

Novel regulatory domains involved in regulation of protein stability, subcellular localization, and activation of Ci

The first allele of *cubitus interruptus* (*ci*) was first isolated in 1933 (Tiniakow and Terentieva, 1933). *Ci* was also identified in a screen for genes involved in embryonic patterning in 1980 (Nusslein-Volhard and Wieschaus, 1980). However, understanding the mechanism of *ci* function had to wait for the advent of molecular genetics. *Ci* was cloned in 1990 and its sequence revealed that it contained a zinc finger domain with homology to the *gli* genes in vertebrates (Orenic et al., 1990). At the time, the Glis had been found to be nuclear proteins that bound to the DNA sequence GACCACCCA (Kinzler and Vogelstein, 1990), raising the possibility that Ci performed a similar function in *Drosophila*.

Evidence has accumulated that Ci is the transcription factor that transduces the Hedgehog (Hh) signal. Expression of Hh targets *patched* (*ptc*) and *wingless* (*wg*) expression in the *Drosophila* embryo requires Ci (Forbes et al., 1993). Repression of *hh* and activation of *ptc* and *decapentaplegic* (*dpp*) in the wing imaginal disc is also Ci dependent (Domínguez et al., 1996). Overexpression of *ci* can activate Hh targets such as *ptc*, which has Gli consensus binding sites in its promoter, and Ci can function as a transcriptional activator of constructs with those same Gli binding sites in yeast (Alexandre et al., 1996). Ci/Gli DNA binding sites have subsequently been identified in other Hh targets such as *wingless* (*wg*) (Von Ohlen et

al., 1997), *dpp* (Hepker et al., 1999; Müller and Basler, 2000), and the vertebrate Hh target *HNF3β* (Sasaki et al., 1997).

Full-length *ci* mRNA is uniformly expressed throughout the anterior compartments of both *Drosophila* embryos and imaginal discs, and Ci protein is translated as a full-length 155kD polypeptide (Ci-155). In the absence of Hh signaling, such as in the far anterior of the wing disc, Ci-155 is proteolytically processed into Ci-75, a smaller 75kD form of Ci that translocates to the nucleus and represses transcription of target genes such as *hh* and *dpp* (Aza-Blanc et al., 1997). In cells adjacent to the A/P border that receive Hh, Ci processing is blocked and Ci-155 accumulates at the border. It is only in these cells that Ci can act as a transcriptional activator (Ci-act) (Aza-Blanc et al., 1997).

Genetic analysis of *ci* function has been complicated by its location on the fourth chromosome. The small size of the fourth chromosome lowers the frequency of recombination events and makes it difficult to separate *ci* alleles from other fourth chromosome mutations. In fact, many Ci genetic studies had used a *ci* allele (Ci^D) that has been found to be a neomorphic mutation that produces a fusion protein of Ci and Pangolin, a transcription factor involved in Wg signaling (Schweizer and Basler, 1998; Von Ohlen and Hooper, 1999). Although interpretation of those studies is complicated by the possibility of unintended crosstalk between the Hh and Wg signaling pathways, subsequent *ci* clonal analysis in the wing imaginal disc using a true *ci* null and a ci^+ rescue construct confirmed

that Ci-75 repressed *hh* and that Ci was required for activation of *ptc* and *dpp* (Méthot and Basler, 1999).

While an absolute requirement for Ci to activate Hh target genes such as *ptc* and *dpp* has been established, the precise form of this transcriptional activator (Ci-act) is still unknown. In contrast to Ci-75, Ci-155 has never been detected in the nucleus without drug treatment to block nuclear export (Chen et al., 1999a; Croker et al., 2006; Méthot and Basler, 2000). Since Ci constructs that cannot form Ci-75 cannot activate target genes without Hh, simply blocking Ci-75 formation is insufficient for activation of target genes and Hh signaling must somehow regulate the conversion of Ci-155 to Ci-act, possibly through posttranslational modification and/or subcellular localization. For example, several changes to Ci, such as removal of PKA phosphorylation sites (Chen et al., 1999b; Chen et al., 1998; Price and Kalderon, 1999; Wang et al., 1999) and deletion of two N-terminal zinc fingers (Croker et al., 2006), block Ci-75 formation and produce constitutively active protein.

In this chapter, two distinct and novel C-terminal domains of full-length Ci are identified and their roles in regulating formation of Ci-act are examined. As diagrammed in Figure 1, both domains are downstream of the only known activation domain identified in Ci, the putative CBP interaction domain (Akimaru et al., 1997). The Δ1352-1398 domain has significant homology to Gli2 and Gli3, and is crucial for transcriptional activation, and

Figure 1: Ci and transgenic Ci constructs with domains required for activation. Ci to scale with N-terminus to the left and C-terminus to the right. Shown are the zinc finger domain (ZnF), nuclear localization signals (NLS), putative cleavage site (CS), repressor formation domain (RFD), a regulatory region (P) containing PKA/GSK3β/CK1 recognition sites, a CBP binding activation domain (CBP AD), a 1160-1352 domain, and a Gli homology domain (GHD). Also shown is a list of transgenic constructs with black bars indicating deleted residues and effect on Ci-75 formation. PKA- is a Ci construct in which four consensus PKA phosphorylation sites have been mutated to alanine residues. All constructs are N-terminally tagged with HA and C-terminally tagged with Myc except CiΔZ2-Myc, which has a single Myc tag inserted at the point of zinc finger deletion (gift of R. Holmgren).

appears to play a role in nuclear export or cytoplasmic retention of Ci. The second domain, Δ1161-1351, has no known sequence homology and seems to be important for blocking transcriptional activation and decreasing protein stability.

Results

Ci C-terminal tags are ineffective in tissue culture and *in vivo*. To characterize Ci-act, a polyclonal rabbit antibody to the C-terminal hundred residues in Ci was generated, and multiple C-terminally tagged Ci constructs were made. However, the effectiveness of these tags and antibodies was very poor; all produced only a very low level of specific signal (data not shown). For example, in a tissue culture experiment in which cells are transfected with equal amounts of DNA, GFP fluorescence is detected in fewer cells and at a lower overall intensity in clone-8 (Cl-8) cells transfected with a Ci-GFP construct (Figure 2A, panel A) compared to Cl-8 cells transfected with GFP, a positive control (Figure 2A, panel B). In contrast, Ci-GFP fluorescence is comparable to GFP fluorescence levels in transfected S2 cells (Figure 2A, panels C & D), indicating that the *UAS-Ci-GFP* construct can make full-length functional protein. The data suggest that the C-terminal GFP epitope in Ci-GFP is either masked or removed in Cl8 cells.

S2 cells lack endogenous Ci and when they are transfected with Ci constructs either in the absence or presence of Hh signaling, they produce a ladder of bands of various molecular weights (see Figure 2B, anti-HA immunoblot). In contrast, Cl-8 cells express endogenous Ci and expression of Ci transgenes produces fewer nonspecific bands, suggesting that Ci behavior in Cl-8 cells may more accurately reflect Ci signaling *in vivo*. In order to unambiguously mark both ends of transgenic Ci constructs, a triple-HA tag was introduced at the N-terminus and a triple-Myc tag was introduced at the C-terminus of Ci to generate

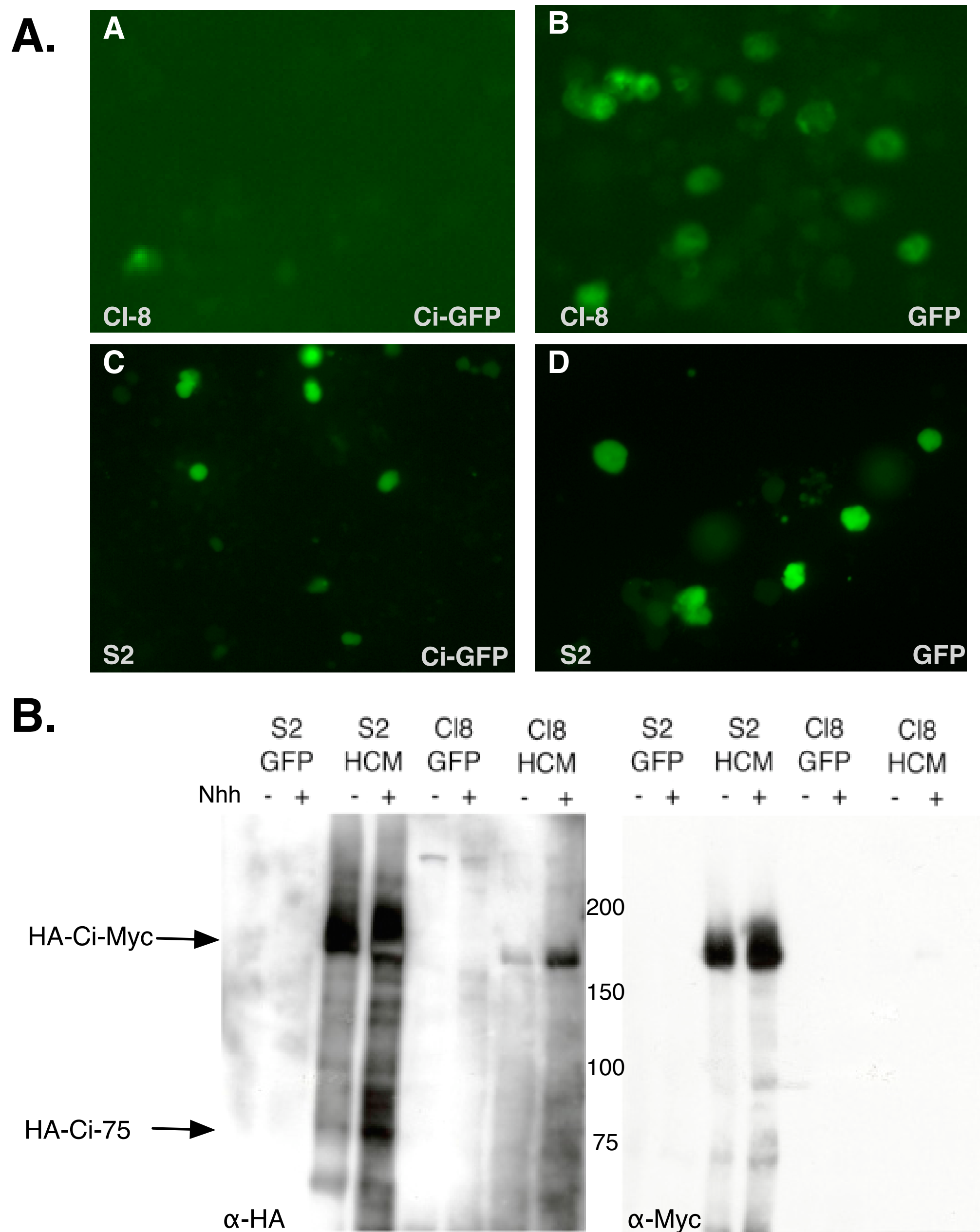


Figure 2: Ci C-terminal tag expression in tissue culture cells. (A) *UAS-Ci-GFP* driven by *actin-GAL4* transfected into Cl-8 cells (panel A) and S2 cells (panel C). As a control for transfection efficiency, *UAS-GFP* driven by *actin-GAL4* were also transfected into Cl-8 cells (panel B) or S2 cells (panel D). (B) Immunoblot of cell lysates expressing either GFP (negative control) or HCM probed with anti-HA (left) or anti-Myc (right).

UAS-HCM (as diagrammed in Figure 1). As shown in Figure 2B, C-terminal tag instability in Cl-8 cells is not limited to GFP tags, since full-length HCM protein bands, which are clearly visible using anti-HA antibodies, are barely detectable when the same lysates are probed using an anti-Myc antibody. Given that the lysates in Figure 2B were separated under denaturing conditions, it seems that C-terminal epitopes are removed from Ci proteins in Cl-8 cells, rather than being masked.

Hh signaling appears to stabilize C-terminal tags on Ci. In order to determine whether the C-terminus of Ci is similarly unstable *in vivo*, *HCM* was expressed in wing discs. *C765-GAL4* drives expression of *UAS-HCM* throughout the wing disc, as indicated by the uniform staining of anti-HA antibody in Figure 3A. In contrast, anti-Myc staining of the same disc is much weaker overall compared to HA staining and lower levels are observed in the anterior compartment compared to the posterior compartment (see Figure 3B). To determine if the decreased anterior anti-Myc staining was due to Ci-75 formation in the anterior, *C765-GAL4* was used to drive expression of *UAS-ΔRFD*, a tagged Ci construct which lacks a Repressor Formation Domain (amino acid residues 713-760). Although HA-Ci-75 formation is blocked in flies expressing ΔRFD, anti-Myc staining is still reduced in the anterior (Figure 3D), which may indicate the existence of a C-terminal modification of Ci distinct from Ci-75 formation.

The pattern of anti-Myc staining in Figure 3 is very similar to the expression pattern of Hh, suggesting that Hh signaling may play a role in stabilizing the Ci C-terminus. Other

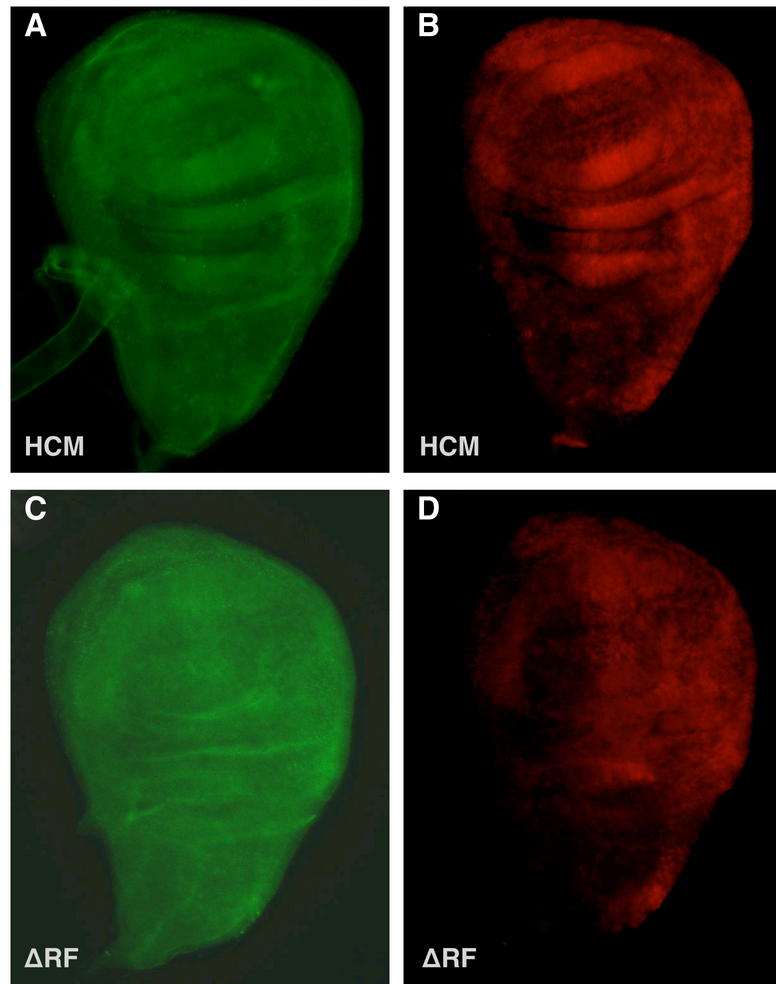


Figure 3: Ci C-terminal tag stabilization in wing disc posterior. *C765-GAL4* drives expression of *UAS-HCM* (panel A) and *UAS- RF* (panel C) throughout the wing disc as shown by rat anti-HA staining (green). However, the C-terminal myc tag as indicated by mouse anti-Myc staining (red) is elevated above background levels in the posterior.

crosses using *ptc-GAL4* and *en-GAL4* drivers to express *UAS-HCM* showed that the stability of the Myc tag was enhanced in the posterior compartment and at the border where Hh signaling is strongest (data not shown). The observation that the C-terminus of Ci appears to be stabilized in regions with high levels of Hh signaling raises the possibility that the C-terminus may play an important role in Ci stabilization.

The C-terminus of Ci contains a domain that is conserved in the Gli proteins. A protein domain in Ci that interacts with CREB Binding Protein (CBP) and was shown to be required for Ci activation *in vitro* was previously mapped to residues 1020-1160 of Ci (Akimaru et al., 1997). It is unlikely that the putative C-terminal modification that is responsible for the low recovery of C-terminal tags extends as far as residues 1020-1160, because cleavage or degradation of the full-length protein so far upstream of the C-terminus should produce a smaller molecular weight isoform and such an isoform has not been observed. It may be relevant that a molecular analysis of Gli3 sequences from patients with Gli3 mutations identified an interesting class of mutations downstream of the CBP that result in a haploinsufficient phenotype (Johnston et al., 2005). Based on these observations, *UAS-ΔC1160-1397*, a deletion construct removing the C-terminal residues 1160-1397 in Ci (Figure 1), was generated.

When the 1160-1397 peptide sequence was used to search the BLAST database, a previously unidentified region of homology to Gli2 and Gli3 was found (Figure 4). While

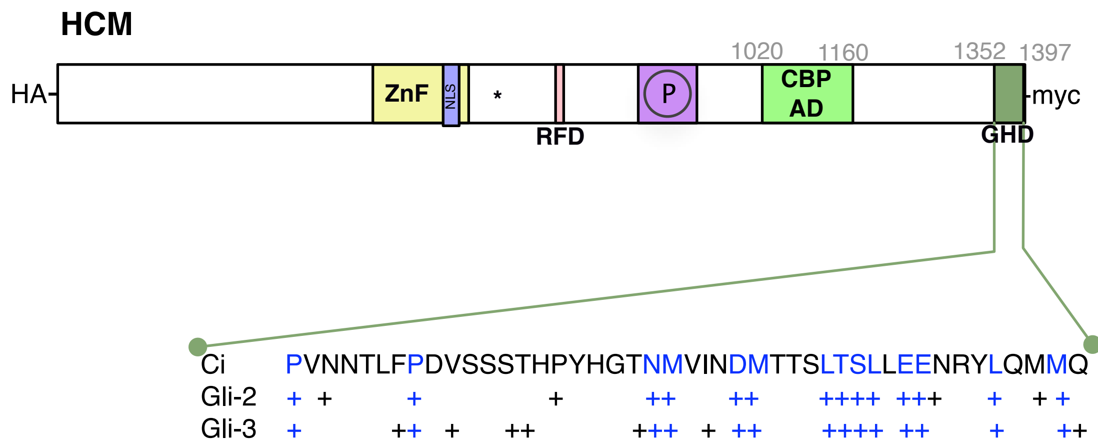


Figure 4: Schematic representation of Ci with sequence identity of Ci, GLI2, GLI3 C-terminal regions. Ci to scale with N-terminus to the left and C-terminus to the right. Shown are the zinc finger domain (ZnF) with individually numbered zinc fingers 1-5, nuclear localization signals (NLS), putative cleavage site (CS), repressor formation domain (RFD), a regulatory region (P) containing PKA/GSK3β/CK1 recognition sites, and CBP binding activation domain (CBP AD). Also shown is the sequence of the Gli Homology Domain (GHD) with identical residues in GLI2 or GLI3 marked with (+). Residues which are identical in all three sequences are highlighted in blue.

there are no regions of homology between residues 1160-1351, the 1352-1397 Ci C-terminus was highly conserved with 40% similarity to Gli2 and 47% similarity to Gli3. Neither Gli2 nor Gli3 act as constitutive transcriptional activators in vertebrates, suggesting perhaps that this domain may play a regulatory role in Ci/Gli activation. Therefore, I also generated an additional construct, *UAS-Δ1352-1397*, that deleted a smaller subset of residues from 1352-1397. The expectation was that expression of both *UAS-ΔC1160-1397* or *UAS-ΔC1352-1397* in the wing disc would result in transgenic proteins with stable C-terminal Myc tags that would not be able to function as transcriptional activators even in the presence of Hh.

ΔC1160-1397 is a constitutively active protein with stable C-terminal tag staining in the absence of Hh. As predicted, the Myc tag is clearly detectable across the wing disc when *UAS-ΔC1160-1397* is driven using *C765-GAL4* (Figure 5H) as compared to ΔRFD, which has decreased levels of anti-Myc staining in the anterior compartment (Figure 5I). However, the phenotype of ΔC1160-1397 in the wing is not what had been expected from overexpression of an inactive protein. As shown in Figure 5D, expression of *ΔC1160-1397* results in overgrowth and ectopic vein formation in both the anterior and posterior compartments (Figure 5A). The posterior compartment phenotype is comparable to the posterior compartment phenotypes resulting from ectopic expression of any Ci protein that can become a transcriptional activator in the presence of Hh, such as HCM (Figure 5B). The overgrowth and ectopic vein formation in the anterior compartment must occur in the absence of Hh signaling. This anterior compartment phenotype is similar to that of

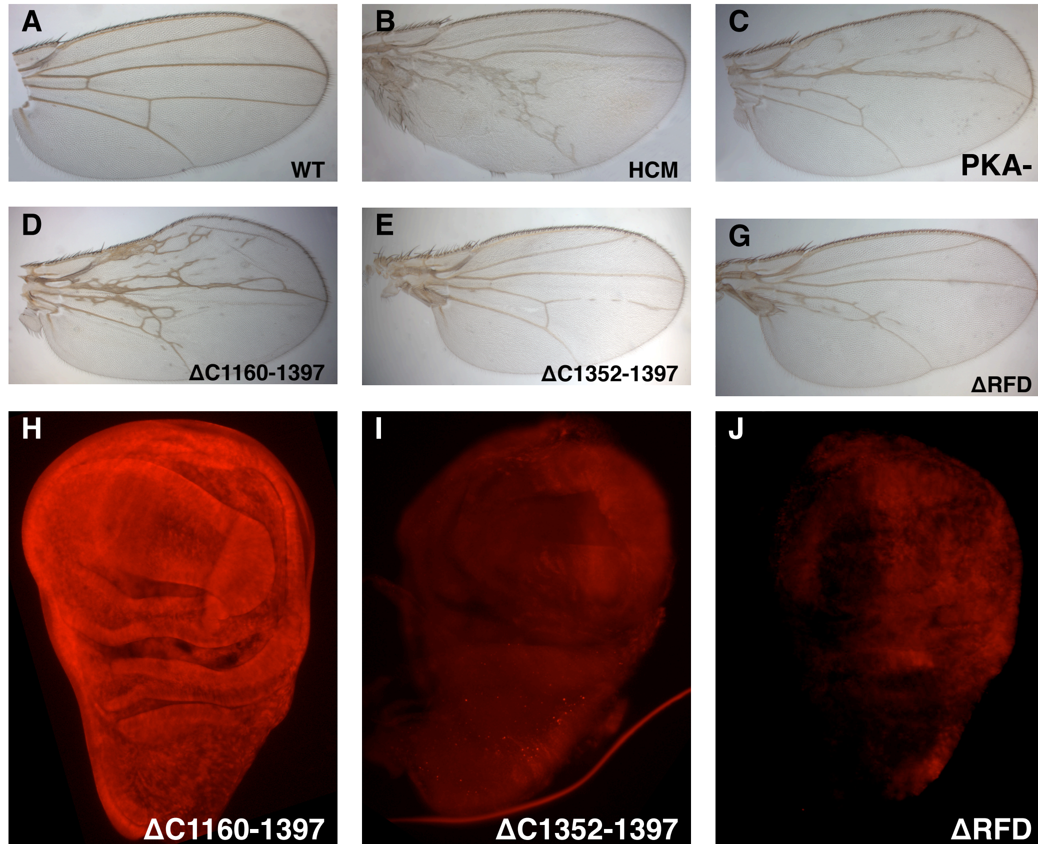


Figure 5: ***In vivo*** **expression of ΔC1160-1397 and ΔC1352-1397.** (A-F) Adult wings from animals expressing Ci constructs driven by *C765-GAL4*. Wings are oriented such that anterior is at the top and posterior is at the bottom. (G-I) Anti-Myc staining of imaginal wing discs in which C-terminal Myc-tagged constructs were expressed throughout the wing disc using the *C765-GAL4* driver. Anterior is to the left and posterior is to the right.

overexpression of *ΔPKA-*, a Ci construct in which four PKA phosphorylation sites (S838A, S856A, S892A, T1006A) have been mutated (Figure 5C). Blocking Ci phosphorylation by PKA has two distinct effects: (1) inhibition of Ci cleavage and (2) prevention of Ci activation (Chen et al., 1999b; Chen et al., 1998; Price and Kalderon, 1999; Wang et al., 1999). Like PKA-, ΔC1160-1397 behaves as a protein that is a transcriptional activator independent of Hh signaling, suggesting that *ΔC1160-1397* contains a motif important for suppressing Ci activation, rather than a motif necessary for activation.

ΔC1352-1397 is Ci protein with decreased C-terminal tag staining in the absence of Hh. Deletion of the Gli homology domain produces a transgenic Ci protein, ΔC1352-1397, that behaves very differently compared to ΔC1160-1397. Like ΔRFD and unlike ΔC1160-1397, ΔC1352-1397 levels are lower in the anterior compartment compared to the posterior compartment as measured by anti-Myc staining when expressed throughout the wing disc using *C765-GAL4* (Figure 5H-I). Unlike ΔC1160-1397, ΔC1352-1397 does not activate transcription in the anterior (Figure 5D-E). Flies expressing *C765-GAL4* driven *UAS–ΔC1352-1397* typically have small interruptions in L4 (Figure 5D) or wings that appear wild-type, and less frequently small amounts of ectopic veination in the posterior compartment. Overall, this posterior compartment phenotype is significantly weaker than the posterior compartment phenotypes of *HCM* (Figure 5B) or *ΔRFD* (Figure 5F) expressing flies. In contrast to the constitutively active ΔC1160-1397, ΔC1352-1397 appears to be less capable of Ci-act formation, even in the presence of Hh.

An *in vivo* transcriptional assay for Ci-act activity. While the wing blade phenotypes are suggestive of differences in Ci protein function, a direct assay of Ci transcriptional activity would provide a more definitive understanding of the apparently contradictory effects of *ΔC1160-1397* and *ΔC1352-1397* expression. Towards this goal, flies expressing various *UAS-Ci* constructs were crossed to *yw,HS-FLP; Actin5C<<CD2<<GAL4*, and early 2nd instar progeny were incubated for 20 minutes at 37°C to generate clones expressing GAL4 protein. Wing discs of the resulting third instar larvae were dissected and costained with anti-HA and anti-Ptc antibodies (Figure 6).

Hh signaling results in upregulation of *ptc* expression in the *Drosophila* embryo (Forbes et al., 1993; Schwartz et al., 1995) and wing imaginal discs (Hepker et al., 1997), as well as in vertebrates (Goodrich et al., 1996; Marigo et al., 1996). This upregulation of *ptc* requires the presence of Ci-act (Domínguez et al., 1996; Méthot and Basler, 1999, 2001); therefore *ptc* expression can be used to assay whether a Ci transgene can become a transcriptional activator. For example, when considering overexpression of a wild-type Ci construct (Figure 6A-B) or Ci constructs in which Ci-75 formation is blocked (Figure 6E-F), *ptc* expression is upregulated in posterior compartment clones, but not in anterior compartment clones. Neither HCM nor ΔRFD can be activated in the anterior where Hh does not signal. In contrast, in clones expressing PKA-, *ptc* is upregulated in both compartments, because PKA phosphorylation normally functions to prevent Ci-act formation.

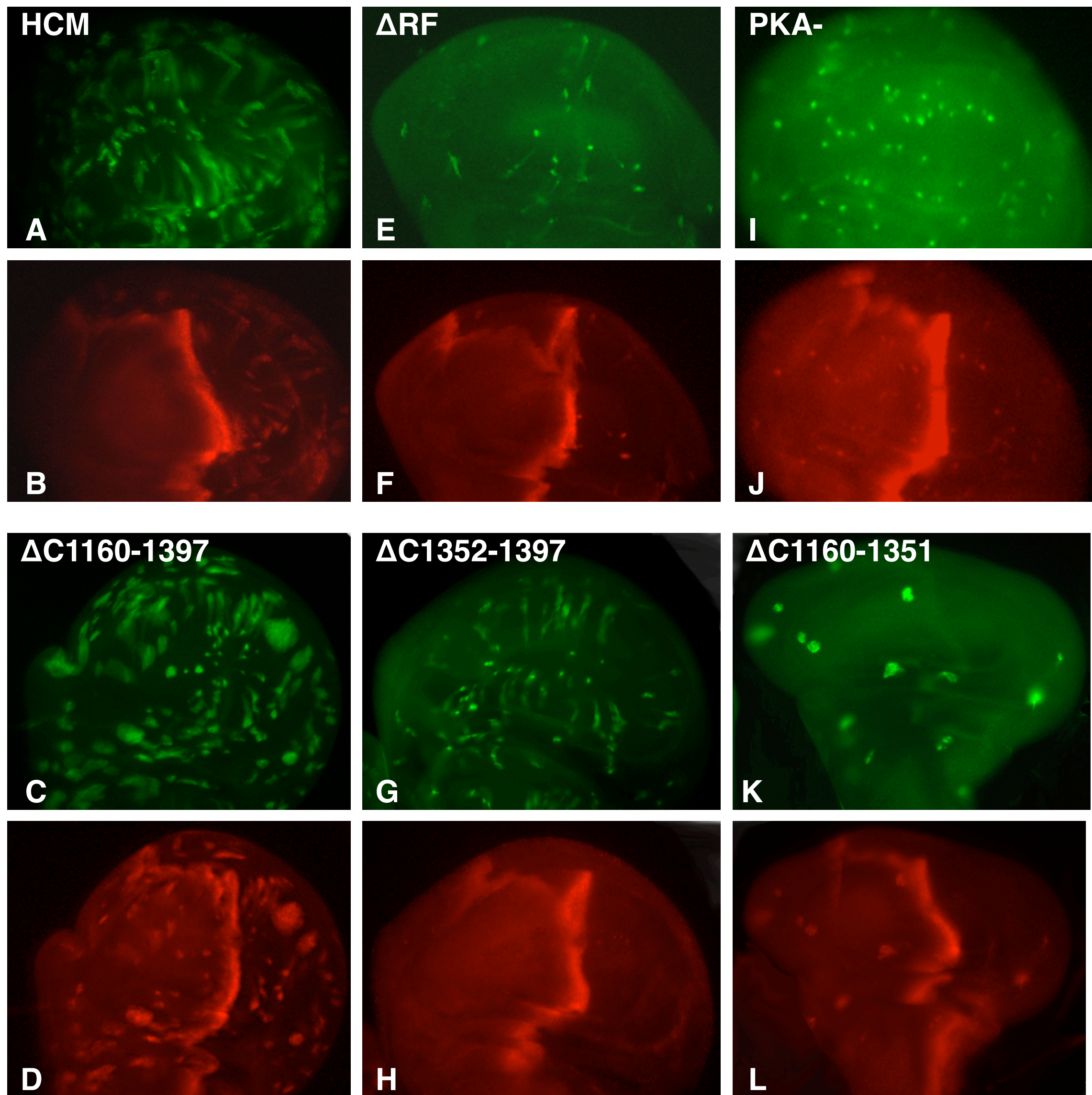


Figure 6: An *in vivo* assay for Ci-act using *ptc.* Imaginal wing discs with clones expressing various HA-tagged Ci constructs were stained with rat anti-HA (in green) to mark the clones. The discs were double-labelled with mouse anti-Ptc (in red) and the stripe of endogenous *ptc* expression marks the A/P border. Anterior is to the left and dorsal is down.

Anti-HA immunoblots of wing disc lysates reveal that expression of *ΔC1160-1397* does not generate appreciable amounts of HA-Ci-75 (Figure 7). This does not explain the strong anterior compartment overgrowth phenotype of ΔC1160-1397 (Figure 5D), because blocking Ci cleavage has no effect in the anterior compartment in the absence of Hh signaling (see Chapter 2). Using the *ptc* activation assay, it is clear that ΔC1160-1397 activates Ci target genes independent of Hh signaling (Figure 6C-D), resulting in overgrowth and ectopic vein formation in both anterior and posterior compartments.

Deletion of the GHD (1352-1397) produces a mutant Ci protein that cannot activate transcription even in the presence of Hh signaling, as evidenced by the observation that none of the *ΔC1352-1397* clones (Figure 6G) ectopically express *ptc* (Figure 6H). One possible explanation for this phenotype is that deletion of 1352-1397 destabilizes protein structure as opposed to specifically affecting Ci activation. However, a combined deletion of 1352-1397 and 1160-1351 produces a protein that can act as a transcriptional activator, which would argue that the GHD specifically prevents Ci-act formation rather than being a determinant of protein structure. Interestingly, deleting residues 1160-1351 alone recapitulates the Hh independent constitutively active phenotype of ΔC1160-1397 (Figure 6K-L).

Residues 1160-1352 have a destabilizing effect on full-length Ci protein. It is apparent in Figure 7 that there is significantly more ΔC1160-1397 protein than HCM in wing disc lysates prepared with an equivalent number of wing discs. To examine the basis

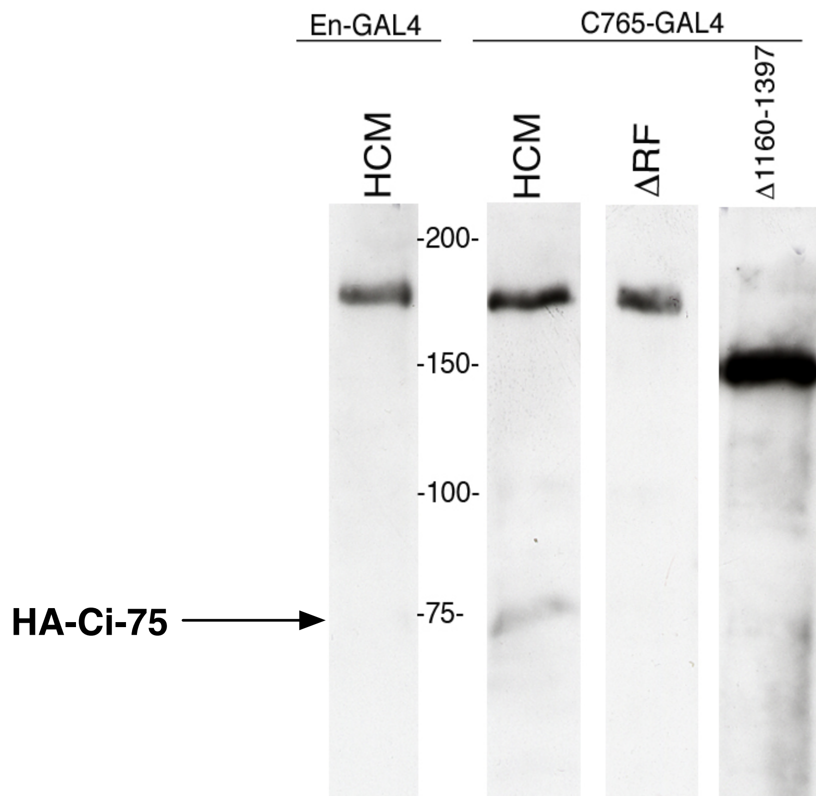


Figure 7: Ci repressor formation is blocked in ΔC1160-1397. Wing disc lysates expressing *C765-GAL4* driven HA-tagged Ci constructs as indicated were separated on a 7.5% SDS-PAGE gel and immunoblotted with rat anti-HA antibody. Despite higher levels of full-length Δ1160-1397, there is not an appreciable amount of HA-Ci-75. ΔRF and *en-GAL4; HCM* were included as negative controls.

for this difference, a S2 cell culture assay using cycloheximide, an inhibitor of protein synthesis, was employed to investigate the effect of Ci residues 1160-1397 on protein stability. As can be seen in Figure 8A, a decline in levels of full length HCM can be detected as early as 6 hours after the addition of cycloheximide. In contrast, significant amounts of ΔC1160-1397 protein continue to persist after 24 hours of cycloheximide treatment. Deletion of the GHD, residues 1352-1397, does not increase stability (Figure 8B), suggesting that the protein destabilization domain resides in the 1160-1351 region. Full-length Ci instability is distinct from Ci-75 formation, because unless these C-terminal residues are deleted in addition, ΔRFD protein is no more stable than HCM protein (Figure 8B). In addition, increased Ci stability is specific to this 1160-1351 region and is not characteristic of all mutations that result in Ci activation. For example, the stability of PKA- protein, which is constitutively active in the absence of Hh signaling, is similar to the protein stability of HCM.

A salivary gland assay for subcellular localization of Ci constructs.

Although Ci-75 has been detected in the nucleus, full-length Ci-155 has never been detected in the nucleus, despite the fact that Ci is required for activation of target genes such as *ptc*. However, assays involving drugs such as leptomycin B, which block nuclear export, have been used in the *Drosophila* imaginal disc (Chen et al., 1999a) and salivary glands (Croker et al., 2006) to show Ci-155 inside the nucleus. In order to determine whether regions 1160-1352 or 1352-1397 are important in subcellular localization of Ci protein, I used a slightly modified version of the salivary gland assay without leptomycin B treatment.

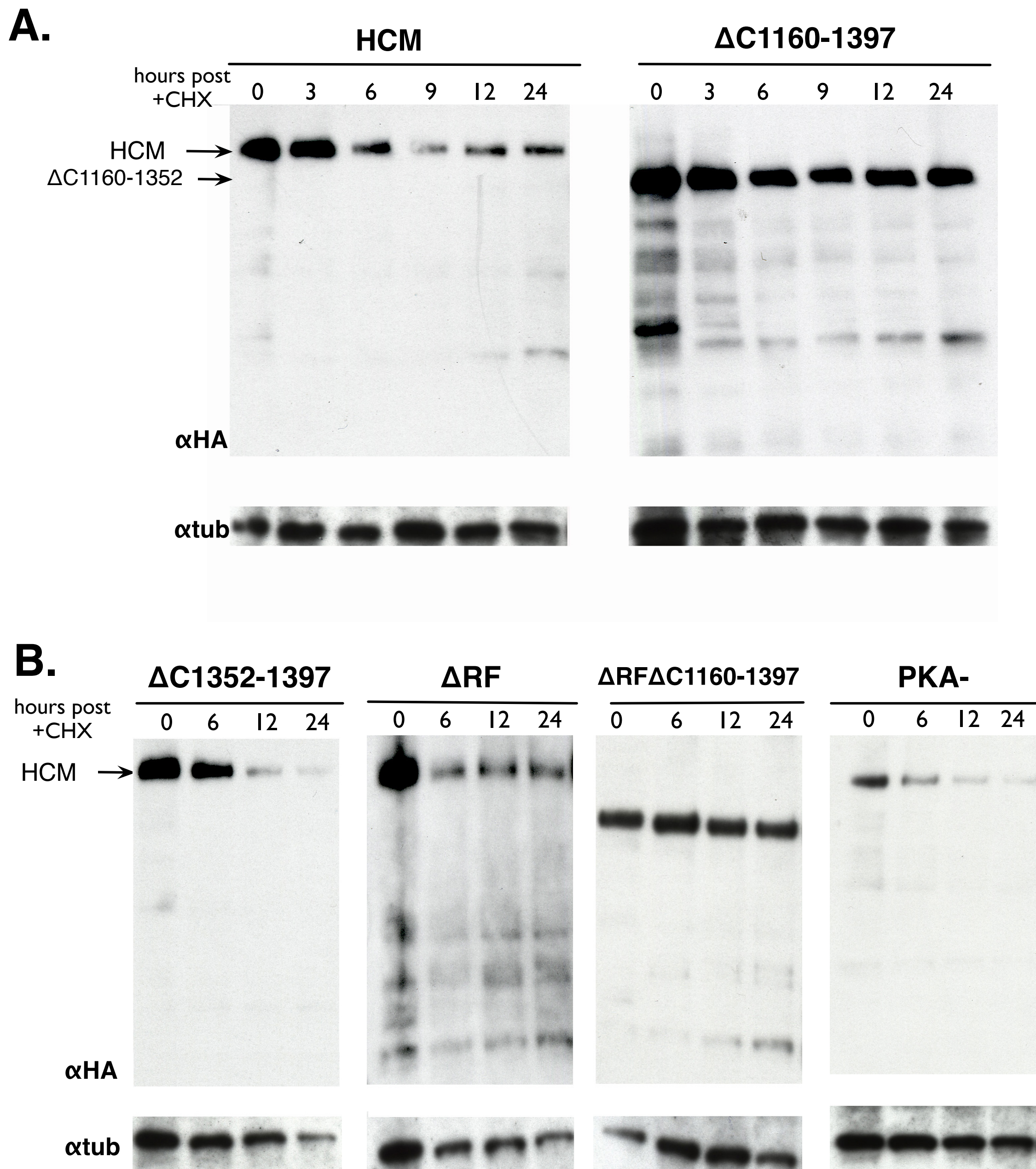


Figure 8: Cycloheximide treatment of S2 cells expressing transgenic Ci constructs. Lysates of S2 cells expressing HA-tagged Ci constructs and treated with cycloheximide (CHX) for times indicated were separated on a 7.5% SDS-PAGE gel and immunoblotted with rat anti-HA antibody and mouse anti-tubulin antibody.

In this assay, *SG3-GAL4* driven expression of tagged wild-type Ci, or HCM, is primarily cytoplasmic and at the cell surface without leptomycin B treatment as visualized using anti-HA antibody staining (Figure 9A). ΔRFD, which cannot make repressor, has a similar expression pattern (Figure 9B). The very faint, vaguely nuclear staining is background signal from the mouse anti-HA antibody used. Staining patterns using anti-Myc antibody to the C-terminal Myc tag were qualitatively similar to the anti-HA staining patterns for all HCM derived constructs discussed in this section, and therefore are not shown herein. However, the signal from Ci C-terminal Myc tags in this assay is quite poor and cannot be attributed to poor anti-Myc antibody staining, since the same antibody detected an internal Myc tag (Figure 9H). The faint anti-Myc staining therefore suggests that the C-terminal instability previously described in tissue culture (Figure 2) and wing discs (Figure 3) is also present in the salivary glands. ΔC713, a Ci construct that is truncated at residue 713, functions as a Ci repressor and is clearly detectable in the nucleus (Figure 9C), consistent with previous reports (Aza-Blanc et al., 1997).

ΔC1160-1397 staining in this assay is quite strong, perhaps reflecting the increased stability of ΔC1160-1397 protein compared to wild-type Ci. Surprisingly, ΔC1160-1397 is clearly visible in the nucleus, even without leptomycin B treatment (Figure 9D). It is unclear why deleting these residues allows detection of Ci in the nucleus, since other mutations that result in activation, such as deletion of the first two zinc fingers in ΔZ2-Myc (Croker et al., 2006), do not have nuclear staining (Figure 9G-H). As an internal control, a construct that

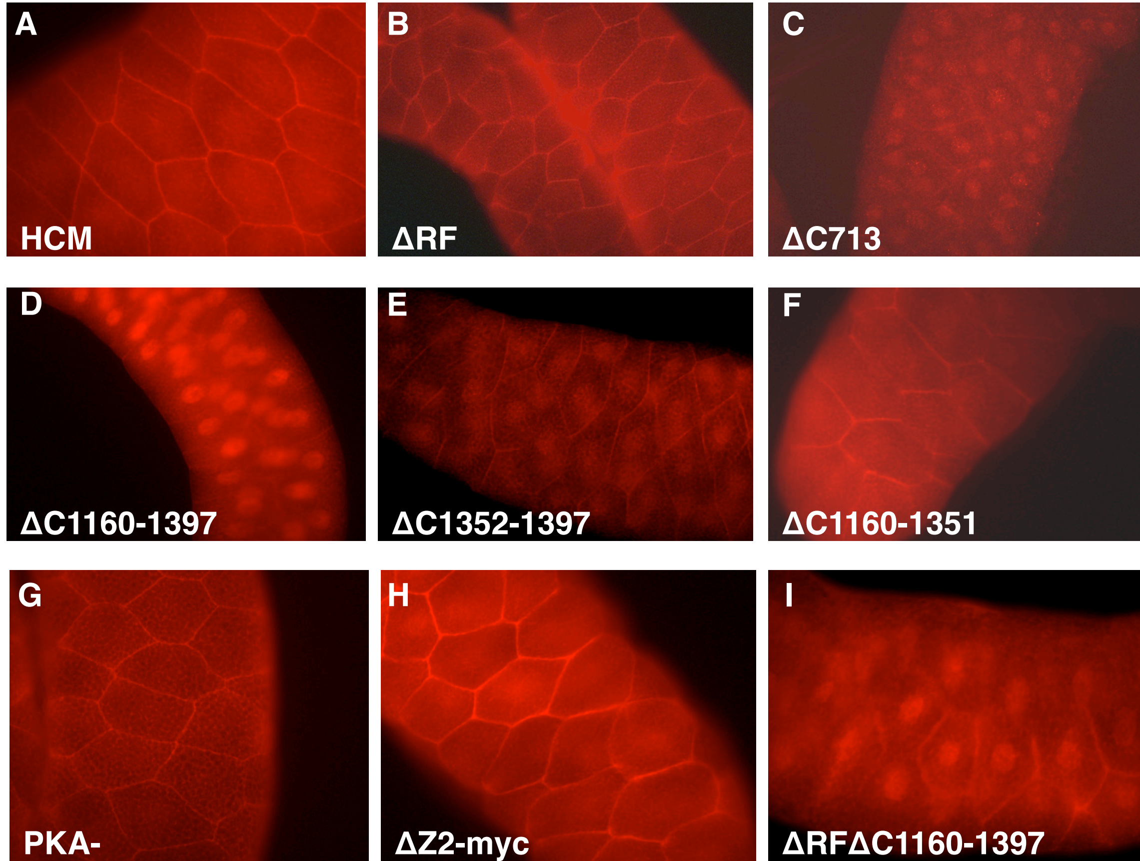


Figure 9: Ci subcellular localization in *Drosophila* salivary glands. Salivary glands expressing various *UAS-Ci* constructs driven using *SG3-GAL4*. All glands were stained with mouse anti-HA (in red) except for the sample in panel H, which was stained with mouse anti-Myc (in red).

is missing both the RFD domain and residues 1160-1397 has enhanced staining in the nucleus (Figure 9F) compared to ΔRFD alone (Figure 9B).

Based on the observation that ΔC1352-1397 cannot act as a transcriptional activator (Figure 5G-H) and ΔC1160-1351 is a constitutively active protein (Figure 5K-L), it was predicted that the inactive protein ΔC1352-1397 would be non-nuclear and that ΔC1160-1351 would be localized to the nucleus in this salivary gland assay. Unexpectedly, the constitutively active ΔC1160-1351 is not found in the nucleus (Figure 9F) while the transcriptionally inactive ΔC1352-1397 is nuclear (Figure 9E). ΔC1352-1397 also has a lower level of staining compared to ΔC1160-1397 because it has the destabilizing residues 1160-1351, indicating that the protein destabilizing domain is limited to the 1160-1351 region.

Discussion

Blocking repressor formation is not sufficient for Ci activation and despite ample experimental evidence for the existence of a distinct form of Ci that functions as the Ci-act, little progress has been made in establishing the precise nature of Ci-act. In this study, I present evidence suggesting that the C-terminus of Ci is modified in the absence of Hh and that blocking this modification may be key to formation of Ci-act. Two novel C-terminal domains of Ci are described that regulate the formation of Ci-act, and the results are summarized in Table 1. Residues 1160-1351 have an inhibitory effect on Ci activation. Deletion of this region produces a mutant Ci protein that is a constitutive transcriptional activator with an increased half-life yet is localized in the cytoplasm or at the cell surface. The other C-terminal domain consisting of residues 1352-1397 has strong sequence homology to Gli2 and Gli3, and is therefore described as a Gli Homology Domain (GHD). Deletion of the GHD produces a mutant Ci protein that is localized to the nucleus, yet is transcriptionally inactive. Based on this data, it appears that the GHD is required for the formation of Ci-act, and contains either a nuclear export signal or a cytoplasmic retention domain. Deletion of both 1160-1351 and the GHD generated a Ci mutant protein that cannot form Ci-75 and is a constitutive transcriptional activator with an increased half-life that is localized in the nucleus.

Current models postulate the existence of a multitude of distinct pools of full-length Ci, such as Ci that will be cleaved to make Ci-75, Ci that is completely degraded by the

Ci Construct	*ptc* Activation	Protein Stability	Nuclear Localization	Ci Repressor Formation
HCM	+	-	-	+
ΔRFD	+	-	-	-
CiΔZ2-myc	+++	n.d.	-	-
PKA-	++	-	-	-
CiΔC1160-1397	+++	+++	+	-
CiΔC1160-1351	+++	+++	-	n.d.
CiΔC1352-1397	0	-	+	n.d.

Table 1: Effects of various Ci mutations. *ptc* activation column indicates construct's ability to activate transcription of the Hh target gene *ptc* in the wing disc. "0" indicates that no *ptc* activation was observed. Protein stability column measures Ci construct stability in tissue culture cycloheximide assay. Nuclear localization column indicates whether Ci construct can be detected in the nucleus in salivary gland cells. Ci repressor formation column indicates whether construct undergoes cleavage to form repressor. n.d.= not determined

proteasome in low Hh situations, Ci that is degraded by the proteasome in high Hh situations, Ci that can be converted Ci-act, Ci-act that is tethered in the cytoplasm, and Ci-act that is in the nucleus. If such distinct pools exist, there should be some way in which they can be distinguished. The low level of detection of Ci C-terminal tags in tissue culture, the wing imaginal disc, and salivary glands documented in this chapter may provide insight into the physical form of Ci-act. The slight increase in stabilization of the C-terminal tag in the posterior was suggestive that the C-terminus is modified in the absence of Hh signaling, although it could not be definitively proven.

I identified a C-terminal domain of Ci (residues 1160-1351) that has an inhibitory effect on Ci activation and appears to regulate the pool of full-length Ci that is targeted for degradation. Deletion of this region produces a Ci protein that is constitutively active, even in the absence of Hh signaling. Although this phenotype is similar to that resulting from removal of the PKA phosphorylation sites in Ci, ΔC1160-1351 is also more stable than wild-type Ci or PKA-, as shown in a tissue culture assay using cycloheximide treatment to block protein synthesis. While there are no clues to its function based on homology or secondary structure predictions (D. Devos, personal communication), given the 1160-1351 region's unique protein destabilizing phenotype, *debra* and *hib/roadkill* are two possible components in the Hh signaling pathway that may interact with this region.

Early studies of activation domains in transcription factors, which are domains required for transcriptional activation that are not involved in DNA binding, found that these domains are typically rich in basic residues (Mitchell and Tjian, 1989). A putative activation domain in Ci was identified at the C-terminus by searching the Ci peptide sequence for this characteristic and replacing this region with a repressor domain. This mutation transformed Ci from a transcriptional activator to a transcriptional repressor of target genes (Alexandre et al., 1996). Given that the *ci* clone used in the Alexandre et al. work appears to be a truncated construct containing only 1,282 amino acids compared to the 1,397 residues expected for full-length Ci, I was unable to ascertain the precise location of this putative activation domain. However, subsequent work with a full-length Ci sequence has shown that Ci contains a C-terminal CBP binding domain at residues 1020-1160 and that CBP is required for Ci to activate Hh target genes *ptc*, *dpp*, *wg* (Akimaru et al., 1997). Therefore, it has been widely accepted that the CBP AD located at residues 1020-1160 is the only known activation domain of Ci.

The GHD discussed in this chapter also appears to be an activation domain. The high level of conservation with Gli2 and Gli3 suggests that the region is crucial for Ci/Gli function and removal of this domain produces a mutant Ci protein that cannot activate transcription of the Hh target *ptc.* However, this domain is neither particularly rich in basic residues (see Figure 4), nor is this domain conserved in Gli1, the only Gli protein that is not cleaved to form repressor and appears to exist only as a transcriptional activator (Dai et al., 1999; von Mering and Basler, 1999). These observations suggest that the GHD is not

necessarily required to recruit transcriptional machinery to enable Ci to activate transcription, since Gli1 can activate transcription without the GHD. An alternative model for how GHD functions to activate Ci is that posttranslational modification of the GHD could convert Ci-155 to Ci-act. This is unlikely because ΔC1160-1397 can activate transcription without the GHD. Another possibility is that the GHD is required to release the inhibitory effect of 1160-1351 and direct Ci-155/Gli2/Gli3 towards a cytoplasmic complex for Ci activation.

Although it is possible that the GHD activates Ci by targeting it to the nucleus, this is unlikely since it is deletion of the GHD that results in nuclear localization of the mutant Ci construct. Many groups have used the large cells of the *Drosophila* salivary glands to assay subcellular localization (Croker et al., 2006; Méthot and Basler, 2000; Sisson et al., 2006; Torroja et al., 2004; Walthall et al., 2007). Because Hh signaling is not normally active in the salivary glands, this assay is artificial. This is especially true of Ci because the salivary glands must be treated with drugs to prevent nuclear export in order to detect any nuclear accumulation. The unexpected result that deletion of the GHD allows the Ci protein to accumulate in the nucleus without drug treatment, would suggest that the GHD has two apparently contradictory functions: (1) it is required for Ci activation and (2) it is required for nuclear export.

In summary, I have shown that the C-terminus of Ci appears to be modified in the absence of Hh and I have identified two adjacent C-terminal regulatory domains in Ci that

regulate the formation of Ci-act. The domain encompassing residues 1160-1351 destabilizes the Ci protein and inhibits the formation of Ci-act. The more distal GHD domain is required nuclear export and for activation of full-length Ci containing the 1160-1351 domain. Further study is required to identify the proteins in the Hh signaling pathway interact with these domains, to analyze the mechanism by which they regulate Ci activation, and to determine if the domains play a role in vertebrate Gli signaling.

Materials and Methods

Molecular biology: The plasmid *pBSKS-Ci-GFP* was made using *pBSKS-KCiD* (gift from P. Aza-Blanc) containing a *ci* cDNA clone that we altered to add a gly-gly-gly linker and GFP tag of D. Casso) at the C terminus of Ci. The plasmid *pBSKS-HCM* was made using *pBSKS-KCiD* (gift from P. Aza-Blanc) containing a *ci* cDNA clone that we altered to add a 3X-HA epitope tag at the N terminus and a 3X-Myc epitope tag at the C terminus of Ci. Triple glycine linkers were also added between each tag and the Ci protein. *pUAST-GFP* and all epitope tags were gifts of D. Casso. *pUAS-Nhh* and *pActin-GAL4* were gifts from G. Ehrenkaufer.

UAS-ΔC1160-1397, *UAS-ΔC1352-1397*, *UAS-ΔC1160-1352*, *UASΔRF*, *UASΔRFΔC1160-1397*, and *UAS-PKA-* were all derived from the *pBSKS-HCM* construct described above, and include an N-terminal 3X-HA tag and a C-terminal 3X-Myc tag. They were all cloned as KpnI-XbaI fragments into pUAST (Brand and Perrimon, 1993) and integrated into the fly genome by germ line transformation.

For PKA-, amino acids S838, S856, S892, and T1006 were mutated into alanine residues using the Quikchange site directed mutagenesis kit (Stratagene). The deletion constructs *UAS-Δ C1160-1397*, *UAS-ΔC1352-1397*, *UAS-ΔC1160-1351*, *UASΔRF*, *UASΔRFΔC1160-1397*, were generated using the Quikchange mutagenesis kit (Stratagene) with the following modifications to the protocol as described below. 5'phosphorylated

primers flanking the region to be deleted were synthesized commercially (GibcoBRL, Operon, Elimbio) such that the forward primer was downstream of the last amino acid to be deleted and the reverse primer was upstream of the first amino acid to be deleted. After eighteen cycles of DNA synthesis using PFUTurbo (Stratagene) , the parent vector was digested using DpnI, the synthesized DNA was ethanol precipitated, resuspended in DNA ligation buffer, ligated for 5 minutes at room temperature using a Rapid Ligation Kit (Roche), and transformed into XL1-Blue supercompetent cells. (Stratagene). Transformants were screened using DNA sequencing and positive clones were sequenced in their entirety to confirm that no second site mutations had been introduced.

Cell culture: *Drosophila* S2 cells were cultured in M3 media (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. *Drosophila* clone-8 (Cl-8) cells were cultured in M3 media (Sigma) supplemented with 2% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.125 U/ml insulin, and 2.5% fly extract. Cell lines were maintained at 24°C under air. Transfections were performed using Effectene (Qiagen), following the manufacturer's protocols.
For conditioned media experiments, 6 day old cultures of S2 cells (as a negative control) or S2 cells expressing *Nhh* were spun down at 700 rpm and the supernatants were passed through a 0.2 µm filter (Millipore). Cells were incubated 48 hours after transfection in a 1:1 ratio of S2 media: conditioned media for 18 hours. For the cycloheximide experiments, cells were treated 48 hours after transfection with 100 µM cycloheximide (Sigma), harvested after

0, 3, 6, 9, 12, or 24 hours of incubation, and stored frozen at -70°C, so that samples could be processed into lysates in parallel.

Fly strains: *C765-GAL4* and *en-GAL4* were obtained from the Bloomington Fly Center. yw, HS-FLP; Actin5C<<CD2<<GAL4 /CKG28 was obtained from F. Hsiung. *SGS3-GAL4* (Cherbas et al., 2003) was obtained from X. Lu. *UAS-ΔZ2-myc* was obtained from R. Holmgren.

Experimental fly crosses: *UAS-Ci* constructs were crossed to either *C765-GAL4*, *SGS3-GAL4,* or *yw, HS-FLP; Actin5C<<CD2<<GAL4 /CKG28* as described (Brand and Perrimon, 1993) at room temperature. For *yw, HS-FLP; Actin5C<<CD2<<GAL4* crosses to *UAS-Ci* constructs, early 2nd instar larvae were incubated for 20 minutes at 37°C to generate clones.

Immunohistochemistry: Cells were transferred to a coated chamber slide (Nalge Nunc), fixed in 4% formaldehyde in PBS, washed with PBS + 0.1% Tween-20 (PBST), and mounted in Vectashield (Vector Laboratories). Wandering 3rd instar larvae were cut in half and everted in *Drosophila* Ringer's solution. Carcasses were fixed in a solution of 8% formaldehyde diluted 1:3 in Brower's fixative (150mM PIPES, 3mM magnesium sulfate, 1.5mM EGTA, 1.5%NP-40, pH 6.9) for 2 hours at 4°C. After washing with PBST for 30 minutes, samples were blocked with 5% normal donkey serum (NDS) in PBS-T for 30 minutes at room temperature and incubated with primary antibody at 4°C for 16 hours. Samples were washed with PBST for 4x 20 minutes and then were incubated with secondary

antibody (Jackson Immunoresearch) for 2 hours at room temperature. After extensive washing with PBST, imaginal discs or salivary glands were dissected from carcasses and were mounted in Vectashield (Vector Laboratories) for microscopic observation. Antibodies used were: rat α-HA (Roche) at 1:200, mouse α-HA (Santa Cruz Biotechnology) at 1:200, mouse α-Myc (Santa Cruz Biotechnology) at 1:200, and α-Ptc mouse monoclonal (gift from I. Guerrero) at 1:150.

Adult wing blade mounts: Wings were removed from adult animals, dehydrated in isopropanol, and mounted in Euparal.

Immunoblotting: The detection of the Ci cleavage product was essentially performed as described (Aza-Blanc et al., 1997). Wandering third instar larvae were collected, rinsed in PBS, cut in half, and everted. Wing imaginal discs were transferred to PBS containing protease inhibitors (Complete; Roche) at 4°C. Discs were spun down in a microcentrifuge at 3000 rpm for 5 minutes at 4°C and resuspended in RIPA lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2) with protease inhibitors (Complete; Roche), DNase (50 μg/ml), and RNase (25 μg/ml) on ice for an additional 5 minutes. Lysates were heated to 95°C for 5 minutes with SDS-PAGE sample buffer added.

Lysates were separated on a 7.5% polyacrylamide gel by SDS-PAGE under reducing conditions and transferred to PVDF membranes using semidry blotting (1 hour at 1

mA/cm^2). Membranes were blocked with 5% dry powdered milk in TBST (TBS + 0.1% Tween-20) overnight at 4C. Membranes were then incubated with mouse anti-HA (1:500; Santa Cruz Biotechnology) or rat anti-HA (1:500; Roche) or mouse anti-tubulin (1:1000; Santa Cruz Biotechnology) for one hour. Membranes were washed 4X for 15 minutes and then incubated with the appropriate peroxidase-labeled secondary antibody (1:5000; Jackson Immunoresearch) for 45 minutes. Membranes were washed again and then visualized using ECL Plus Western Blotting Immunodetection System (GE Healthcare) and exposure to Kodak Biomax Light Film (Kodak).

Chapter 4

Discussion

During development, cell fate, patterning, and axis specification in an individual cell are determined by extracellular signals, such as Hedgehog (Hh), received from other cells. The program of spatial and temporal gene expression initiated upon reception of the Hedgehog signal is regulated by the transcription factor Cubitus interruptus (Ci), which binds to regulatory DNA sequence elements and modulates expression of target genes such as *patched* (*ptc*), *decapentaplegic* (*dpp*), and *hh* itself. My work has focused on understanding how posttranslational modification of Ci affects Ci function in Hh signaling. Multiple posttranslational mechanisms regulate Ci activity and the work described here identifies and analyzes three distinct regulatory domains of Ci. One domain, the RFD (Repressor Formation Domain), is required for Ci repressor (Ci-75) formation. A second domain, comprising residues 1160-1351, is involved in destabilizing Ci protein and thereby preventing its transformation into Ci activator (Ci-act). The third domain, the Gli Homology Domain (GHD) is crucial for both Ci activation and nuclear export.

In the current model for Ci-75 formation, in the absence of Hh full-length Ci-155 is phosphorylated by PKA/GSK3β/CKI, hyperphosphorylated Ci-155 is recognized by Slimb, which targets Ci for ubiquitination, and the ubiquitinated Ci-155 is specifically cleaved by the proteasome to release Ci-75. There have been few instances of specific cleavage by the proteasome, with the processing of NF-κB p105 to NF-κB p50 being the most well-studied

(Palombella et al., 1994). Recent work determined that specific cleavage by the proteasome requires a bipartite signal consisting of a tightly folded domain and a simple sequence region (SSR) (Piwko and Jentsch, 2006). It has been proposed that in the case of Ci, the last three zinc fingers comprise the folded domain and the region 611-712 is the SSR required for Ci-75 formation by the proteasome (Tian et al., 2005).

While there has been data consistent with the model that Slimb targets Ci-155 to the proteasome to produce Ci-75 direct cleavage (reviewed in Jiang, 2006), there is also data that does not fit this model. For example, I have shown that deletion of this putative SSR does not block Ci-cleavage, whereas deletion of the RFD (a very short sequence that is not of particularly low complexity) did block Ci-75 formation. Furthermore, there is no obvious consensus Slimb binding motif in Ci and polyubiquitinated forms of Ci are not detected when Ci processing is blocked using a proteasome inhibitor like MG132 (Chen et al., 1999a).

A sequence homologous to the RFD could not be found in the NF-κB sequence, suggesting that Ci-75 is generated *in vivo* in a process that is not homologous to NF-κB p50 formation. While ubiquitination is commonly associated with protein degradation, there is growing evidence that it can also play a role in endosomal sorting of receptors, transcription factor inhibition (without degradation), and nuclear export (reviewed in Mukhopadhyay and Riezman, 2007). Interestingly, one group has shown that *slimb* is actually upstream of *smo* in

the Hh signaling pathway (Theodosiou et al., 1998), rather than being downstream of *smo* as would be expected in the Slimb-proteasome model.

If the proteasome is not the Ci protease, how would *slimb* function to promote Ci-75 formation? One possible model that does not include proteasomal generation of Ci is illustrated in Figure 1A. In this model, Ci-155 is recognized by Slimb in the absence of Hh signaling and monoubiquitination targets it away from the degradation pathway. Instead, monoubiquitinated Ci is recognized by a protease X that generates Ci-75 in a process that requires the RFD domain. Alternatively, Slimb could act on an inhibitor of Ci cleavage rather than on Ci itself (see Figure 1B). In this model, Smo inhibits Ci-75 formation, possibly by recruiting the Cos/Fu/Ci complex to the membrane and away from cytoplasmic determinants required for Ci processing. In the absence of Hh signaling, Slimb targets Smo for endocytosis and degradation, thus freeing the Cos/Fu/Ci complex so that Ci can be phosphorylated by PKA/GSK3β/CKI. Hyperphosphorylated Ci is then cleaved by the Ci protease in a process that requires the RFD. In the presence of Hh, Smo is modified such that it cannot interact with Slimb, stabilizing the Smo protein at the cell membrane and inhibiting Cos/Fu/Ci complex from catalyzing Ci proteolysis.

In the absence of Hh signal, PKA has a dual role as a promoter of Ci-75 formation and repressor of Ci activation. When PKA phosphorylation sites are mutated in Ci, the mutant Ci protein does not make Ci-75 and acts as a constitutive activator (Chen et al., 1999b). This result suggests that PKA phosphorylation targets Ci-155 for cleavage while

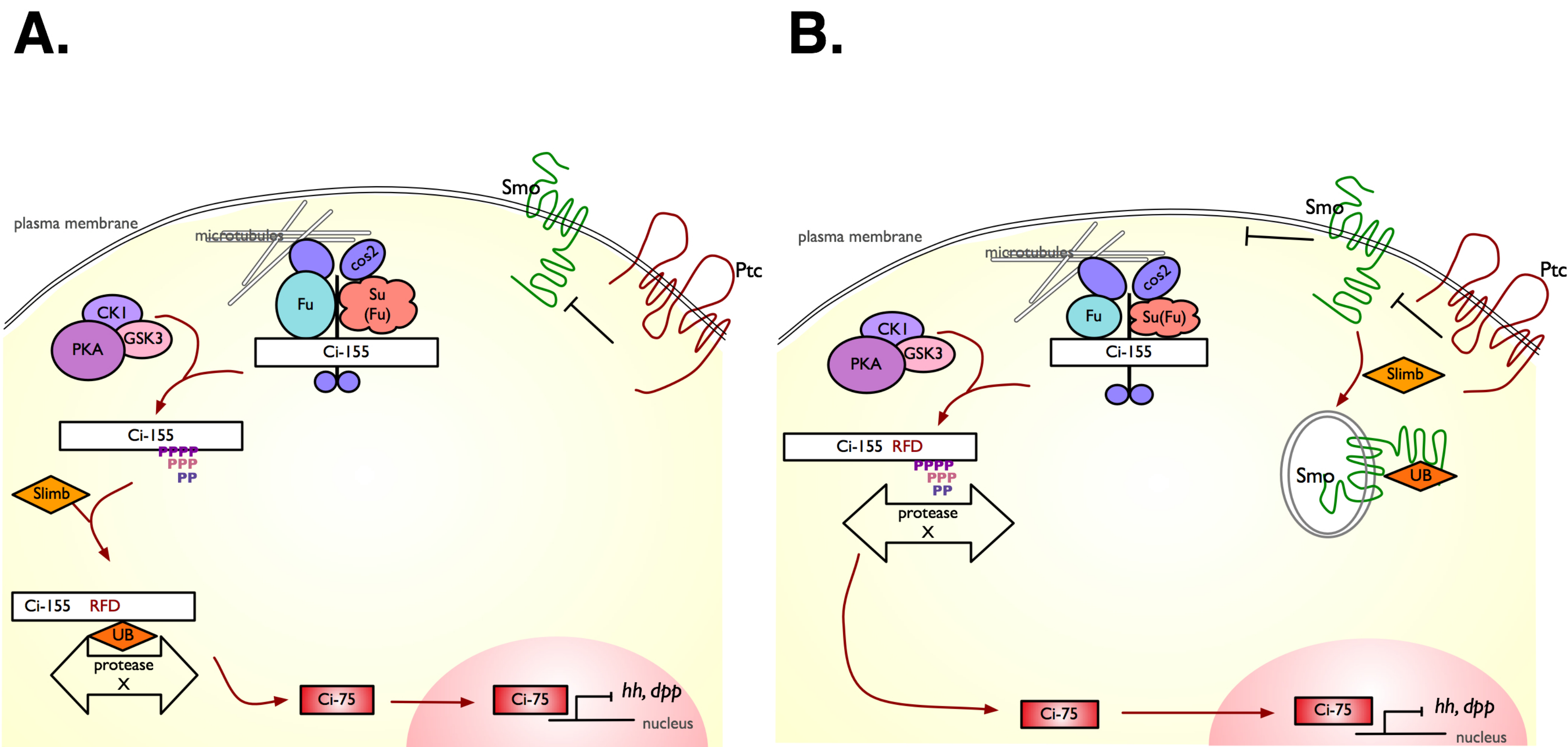


Figure 1: Alternative models for Slimb's role in Ci-75 Formation. (A) Slimb targets Ci-155 for monoubiquitination and cleavage by an unidentified nonproteasomal protease, protease X. (B) Smo functions to inhibit Ci-75 formation and Slimb targets Smo for ubiquitination and endocytosis in the absence of Hh.

simultaneously preventing it from associating with complexes required for Ci-act formation. In contrast to mutation of the PKA sites in Ci, deletion of the RFD does not result in Ci activation. If the RFD is the recognition site for the Ci protease, removal of the site would prevent Ci-75 formation without having an effect on Ci activation, since the protein would still be phosphorylated by PKA and thereby blocked from activation. Importantly, *slimb* loss of function clones also result in Ci activation and inhibition of Ci-75 formation (Jiang and Struhl, 1998). This behavior is not consistent with a model in which Slimb targets full-length Ci for cleavage to form repressor, since this model would suggest that removal of *slimb* would block cleavage without having an effect on Ci activation. *slimb*'s Ci activation phenotype instead supports a model in which Slimb targets a component upstream of both Ci and PKA, and that Slimb functions to inhibits Ci-75 formation without acting on Ci directly.

My observation that deletion of 611-712 generates a slightly smaller than expected Ci cleavage product raises the possibility that the RFD acts as a molecular ruler. In this model, the proteasome or a nonproteasomal exo-site Ci protease would recognize the RFD and cut Ci at a site at a distance from the RFD (Figure 2). While this hypothesis awaits testing, it is interesting to note that similar experiments to map cleavage sites in Gli2 and the *Aspergillus nidulans* Ci homologue, PacC, also generated cleavage products that were not the expected size. These data are consistent with my observation that the Ci site that determines cleavage is distinct from the cleavage site itself.

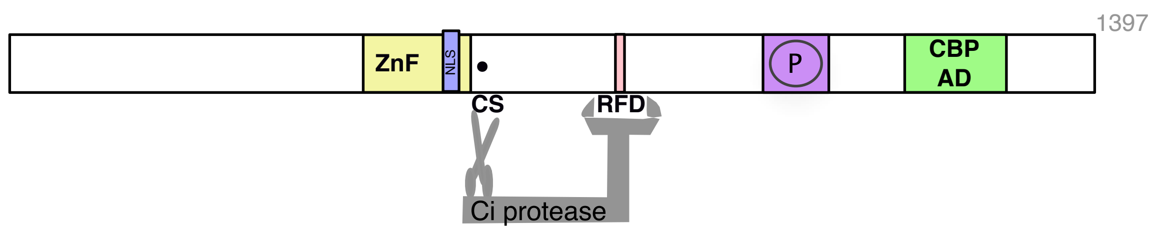


Figure 2: Model of exo-site cleavage of Ci. Schematic representation of Ci with N-terminus to the left and C-terminus to the right. Shown are the zinc finger domain (ZnF), nuclear localization signals (NLS), putative cleavage site (CS), repressor formation domain (RFD), a regulatory region (P) containing PKA/GSK3β/CK1 recognition sites, and a CBP binding activation domain (CBP AD). The Ci protease has one domain that recognizes Ci at the RFD and another domain containing an active site which cleaves Ci at a site 8kD upstream of the RFD recognition site.

Ci-75 is produced by a complex process with multiple regulated steps. Many questions remain about Ci-75 formation and little is known about its targets. Ci's location on the fourth chromosome makes it difficult to determine the specific contribution of Ci-75 to Hh signaling, as it has not been technically feasible to genetically remove Ci-75 by replacing wild-type Ci with a ΔRFD mutant. Further study is needed to identify novel regulators of Ci-75 formation, elucidate the mechanism of the RFD, and identify the targets of Ci-75.

In Chapter 2, I present evidence suggesting that Ci-75 production is relatively inefficient and I show that blockage of the cleavage event that forms Ci-75 is insufficient for Ci activation. Immunostaining with the 2A1 monoclonal antibody to Ci, which recognizes an epitope C-terminal to the zinc finger domain, reveals low levels of full-length Ci in the anterior compartment away from the border as well as elevation of full-length Ci at the border (Aza-Blanc et al., 1997). It is therefore apparent that mechanisms other than Ci repressor formation exist to prevent full-length Ci from activating transcription in the absence of Hh signaling. In Chapter 3, I show that the C-terminus of wild-type Ci can be detected in cells that express or receive Hh, but is present at much reduced levels in the absence of Hh. These observations suggest that modification of the C-terminus may be involved in targeting Ci-155 away from the process that converts it to Ci-act. A simple explanation is that C-terminal instability reduces basal levels of Ci and thereby reduces formation of Ci-act. A more interesting possibility is that sequences in the C-terminus are required for and involved in Ci-155's conversion to Ci-act.

I identified two novel C-terminal domains that appear to regulate Ci-activation in distinct ways. The first domain, comprising residues 1160-1351, is involved in destabilizing Ci protein and thereby preventing its transformation into Ci activator (Ci-act). The second domain, the Gli Homology Domain (GHD) was found to be crucial for both Ci activation and nuclear export. Current models for Ci activation postulate the existence of multiple pools of Ci in distinct complexes and at various stages of activation (Figure 3). In such a model, the 1160-1351 region could mediate degradation of Ci-155 by Debra (at low Hh concentrations) or degradation of Ci-act by Rdx (at high Hh concentrations). The GHD domain could be a transcriptional activation domain that is also required for nuclear export and interaction with Suppressor of Fused (Su(Fu)), thus neatly preventing overactivation of Hh signaling by downregulating the nuclear concentration of Ci-act. However, it is difficult to explain all the results in Chapter 3 using the Ci pool model. For example, according to this model, deletion of both domains in ΔC1160-1397 should result in large amounts of a transcriptionally inactive protein localized to the nucleus. Instead, ΔC1160-1397 produces a constitutive activator, suggesting that the GHD actually functions to relieve inhibition of Ci-act induced by the 1160-1351 domain.

Work on the *Aspergillus* homolog of Ci, PacC, supports a model in which PacC self associates in a pH dependent manner to mask a domain required for processing (reviewed in Arst and Penalva, 2003). In Figure 4, I present an alternative model for how these Ci domains regulate Ci activation based on the PacC intramolecular association model. This model is consistent with all of the data discussed in Chapter 3 and summarized in Table 1 of

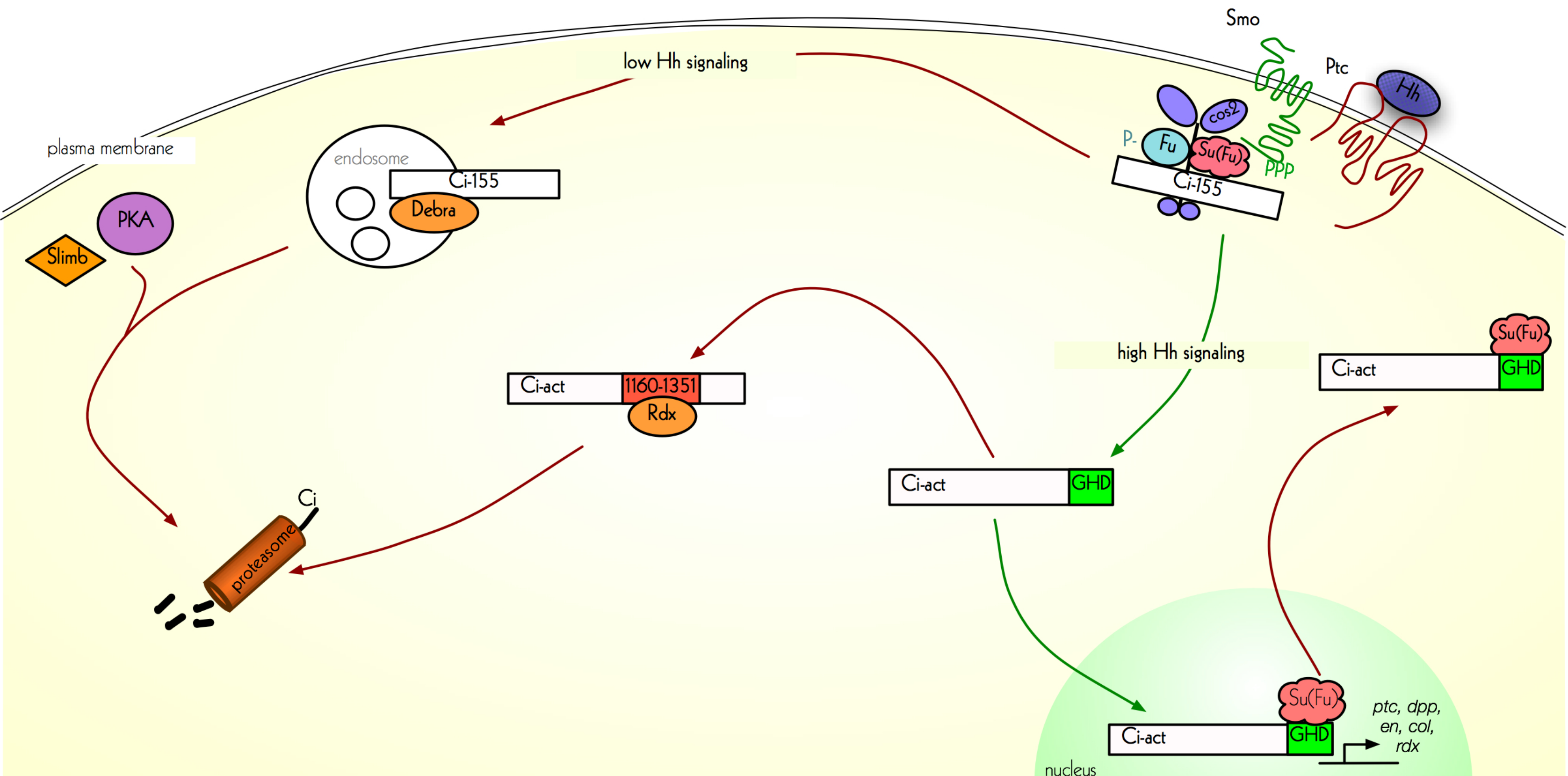


Figure 3: Model for Ci modification and cellular localization in the presence of Hh. Upon binding of Hh to Ptc, Smo is phosphorylated and stabilized at the cell surface, where it prevents the Cos/Fu/Ci/Su(Fu) complex from associating to microtubules and blocks Ci-75 formation. Multiple pools of Ci exist in various stages of modification. In regions of low Hh signaling, full-length Ci-155 is found in endosomes where Debra targets it for degradation in a process that requires PKA and Slimb activity. At high Hh concentrations, Ci-155 is transformed into Ci-act in a process that requires the GHD. Ci-act is either targeted for degradation by Rdx through a direct interaction with the 1160-1351 domain or is transported to the nucleus where it can activate transcription of target genes. The GHD is also required, together with Su(Fu), for nuclear export of Ci.

Figure 4: Ci self-association model. In the absence of Hh, Ci is targeted for degradation by either Debra or Rdx. The C-terminus of Ci is modified such that the GHD cannot inhibit the 1160-1351 domain. This allows 1160-1351 to interacts with first two zinc fingers of Ci, inhibiting Ci activation by blocking CBP AD function. Upon binding of Hh to Ptc, Smo is phosphorylated and stabilized at the cell surface, where it prevents Cos/Fu/Ci/Su(Fu) complex from associating to microtubules and blocks Ci-75 formation. The GHD remains intact and relieves 1160-1351 mediated repression of Ci-act formation. Ci-act translocates to the nucleus where it activates transcription of Hh target genes. The GHD Nuclear Export Signal (NES) and Su(Fu) export Ci-act out of the nucleus.

that chapter. In this model, full-length Ci self-associates through an interaction between the first two zinc fingers (Zf2) and the 1160-1351 domain. This intramolecular interaction masks the CBP activation domain. The GHD has two functions: (1) it blocks the Zf2/1160-1351 interaction, and (2) it is a nuclear export signal (NES). In the absence of Hh, a C-terminal modification occurs such that the GHD cannot disrupt the Zf2/1160-1351 interaction and the activation domain is inaccessible. (This modification also coincidentally removes any tags attached to the Ci C-terminus.) When a cell receives the Hh signal, the C-terminus is not modified, the intact GHD disrupts the Zf2/1160-1351 interaction, thereby unmasking the activation domain. Ci-act then translocates to the nucleus, where it activates transcription of Hh target genes, but is quickly exported out of the nucleus via the GHD domain to prevent hyperactivation of the pathway.

Misregulation of Ci activity can result in developmental defects and cancer. Therefore, the Hh signaling pathway has evolved multiple layers of posttranslational regulation of Ci (such as repressor formation, protein degradation, and subcellular localization) to prevent both underactivation and hyperactivation of transcription and ensure proper patterning. To summarize, my studies of repressor formation and transcriptional activation of Ci identified three distinct domains that further our understanding of how signals such as Hh that are received at the cell surface become transcriptional readouts in the nucleus. One domain, the RFD (Repressor Formation Domain), is required but not sufficient for Ci repressor (Ci-75) formation. I show that the RFD is distinct from the site of cleavage and has homology to the vertebrate Gli proteins

that are cleaved, Gli2 and Gli3. My results also support an alternative model for Ci-75 formation in which Slimb targets Smo for degradation, allowing cleavage of Ci by an exo-site protease. I show that a second domain, comprising residues 1160-1351, is involved in destabilizing Ci protein, regulating Ci turnover, and thereby preventing its transformation into Ci activator (Ci-act). Finally, I identified a third domain, the Gli Homology Domain (GHD) and demonstrate that it is crucial for both Ci activation and nuclear export.

References

Akimaru, H., Chen, Y., Dai, P., Hou, D., Nonaka, M., Smolik, S., Armstrong, S., Goodman, R., and Ishii, S. (1997). Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signalling. Nature *386*, 735-738.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell *86*, 221-232.

Alexandre, C., Jacinto, A., and Ingham, P.W. (1996). Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. Genes Dev *10*, 2003-2013.

Arst, H.N., and Penalva, M.A. (2003). pH regulation in Aspergillus and parallels with higher eukaryotic regulatory systems. Trends Genet *19*, 224-231.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., and Kornberg, T.B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell *89*, 1043-1053.

Bellaiche, Y., The, I., and Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. Nature *394*, 85-88.

Blair, S.S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. Development *115*, 21-33.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K., Dickson, B., and Basler, K. (2000). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell *99*, 803-815.

Chen, C.H., von Kessler, D.P., Park, W., Wang, B., Ma, Y., and Beachy, P.A. (1999a). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. Cell *98*, 305-316.

Chen, Y., Cardinaux, J.R., Goodman, R.H., and Smolik, S.M. (1999b). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. Development *126*, 3607-3616.

Chen, Y., Gallaher, N., Goodman, R.H., and Smolik, S.M. (1998). Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus. Proc Natl Acad Sci U S A *95*, 2349-2354.

Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. Cell *87*, 553-563.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., and Cherbas, P. (2003). EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue. Development *130*, 271-284.

Chuang, P., and McMahon, A. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature *397*, 617-621.

Ciechanover, A., and Schwartz, A.L. (1998). The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. Proc Natl Acad Sci U S A *95*, 2727-2730.

Croker, J.A., Ziegenhorn, S.L., and Holmgren, R.A. (2006). Regulation of the Drosophila transcription factor, Cubitus interruptus, by two conserved domains. Dev Biol *291*, 368-381.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J Biol Chem *274*, 8143-8152.

De Robertis, E.M. (2006). Spemann's organizer and self-regulation in amphibian embryos. Nature reviews *7*, 296-302.

Denef, N., Neubüser, D., Perez, L., and Cohen, S. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell *102*, 521-531.

Desbordes, S.C., and Sanson, B. (2003). The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of Drosophila. Development *130*, 6245-6255.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., and Hui, C.C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development *125*, 2533-2543.

Domínguez, M., Brunner, M., Hafen, E., and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein Cubitus interruptus. Science (New York, NY) *272*, 1621-1625.

Eaton, S., and Kornberg, T.B. (1990). Repression of ci-D in posterior compartments of Drosophila by engrailed. Genes Dev *4*, 1068-1077.

Forbes, A.J., Nakano, Y., Taylor, A.M., and Ingham, P.W. (1993). Genetic analysis of hedgehog signalling in the Drosophila embryo. Dev Suppl, 115-124.

Gallet, A., Angelats, C., Kerridge, S., and Therond, P.P. (2000). Cubitus interruptus-independent transduction of the Hedgehog signal in Drosophila. Development *127*, 5509-5522.

Goodrich, L., Johnson, R., Milenkovic, L., McMahon, J., and Scott, M. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes & development *10*, 301-312.

Hepker, J., Blackman, R.K., and Holmgren, R. (1999). Cubitus interruptus is necessary but not sufficient for direct activation of a wing-specific decapentaplegic enhancer. Development *126*, 3669-3677.

Hepker, J., Wang, Q.T., Motzny, C.K., Holmgren, R., and Orenic, T.V. (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. Development *124*, 549-558.

Hidalgo, A., and Ingham, P. (1990). Cell patterning in the Drosophila segment: spatial regulation of the segment polarity gene patched. Development *110*, 291-301.

Hooper, J.E., and Scott, M.P. (1989). The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. Cell *59*, 751-765.

Hui, C.C., Slusarski, D., Platt, K.A., Holmgren, R., and Joyner, A.L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. Dev Biol *162*, 402-413.

Ingham, P.W., and Hidalgo, A. (1993). Regulation of wingless transcription in the Drosophila embryo. Development *117*, 283-291.

Ingham, P.W., Taylor, A.M., and Nakano, Y. (1991). Role of the Drosophila patched gene in positional signalling. Nature *353*, 184-187.

Jia, J., Amanai, K., Wang, G., Tang, J., Wang, B., and Jiang, J. (2002). Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. Nature *416*, 548-552.

Jia, J., Tong, C., and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. Genes & development *17*, 2709-2720.

Jia, J., Zhang, L., Zhang, Q., Tong, C., Wang, B., Hou, F., Amanai, K., and Jiang, J. (2005). Phosphorylation by double-time/CKIepsilon and CKIalpha targets cubitus interruptus for Slimb/beta-TRCP-mediated proteolytic processing. Dev Cell *9*, 819-830.

Jiang, J. (2006). Regulation of Hh/Gli Signaling by Dual Ubiquitin Pathways. Cell Cycle *5*.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature *391*, 493-496.

Johnson, R., Milenkovic, L., and Scott, M. (2000). In vivo functions of the patched protein: requirement of the C terminus for target gene inactivation but not Hedgehog sequestration. Molecular cell *6*, 467-478.

Johnston, J., Olivos-Glander, I., Killoran, C., Elson, E., Turner, J., Peters, K., Abbott, M., Aughton, D., Aylsworth, A., Bamshad, M.*, et al.* (2005). Molecular and clinical analyses of Greig cephalopolysyndactyly and Pallister-Hall syndromes: robust phenotype prediction from the type and position of GLI3 mutations. American journal of human genetics *76*, 609-622.

Kang, S., Graham, J.M., Jr., Olney, A.H., and Biesecker, L.G. (1997). GLI3 frameshift mutations cause autosomal dominant Pallister-Hall syndrome. Nat Genet *15*, 266-268.

Kasper, M., Regl, G., Frischauf, A.M., and Aberger, F. (2006). GLI transcription factors: mediators of oncogenic Hedgehog signalling. Eur J Cancer *42*, 437-445.

Kent, D., Bush, E.W., and Hooper, J.E. (2006). Roadkill attenuates Hedgehog responses through degradation of Cubitus interruptus. Development *133*, 2001-2010.

Kinzler, K.W., Ruppert, J.M., Bigner, S.H., and Vogelstein, B. (1988). The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature *332*, 371-374.

Kinzler, K.W., and Vogelstein, B. (1990). The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol Cell Biol *10*, 634-642.

Krishnan, V., Elberg, G., Tsai, M.J., and Tsai, S.Y. (1997a). Identification of a novel sonic hedgehog response element in the chicken ovalbumin upstream promoter-transcription factor II promoter. Molecular endocrinology (Baltimore, Md *11*, 1458-1466.

Krishnan, V., Pereira, F.A., Qiu, Y., Chen, C.H., Beachy, P.A., Tsai, S.Y., and Tsai, M.J. (1997b). Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. Science *278*, 1947-1950.

Krishnaswamy, S. (2005). Exosite-driven substrate specificity and function in coagulation. J Thromb Haemost *3*, 54-67.

Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., and Cohen, S.M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. Nature *381*, 387-393.

Lessing, D., and Nusse, R. (1998). Expression of wingless in the Drosophila embryo: a conserved cis-acting element lacking conserved Ci-binding sites is required for patched-mediated repression. Development *125*, 1469-1476.

Levin, M., Johnson, R.L., Stern, C.D., Kuehn, M., and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell *82*, 803-814.

Lewis, E.B. (1978). A gene complex controlling segmentation in Drosophila. Nature *276*, 565-570.

Lewis, K., Drossopoulou, G., Paton, I., Morrice, D., Robertson, K., Burt, D., Ingham, P., and Tickle, C. (1999). Expression of ptc and gli genes in talpid3 suggests bifurcation in Shh pathway. Development (Cambridge, England) *126*, 2397-2407.

Liu, Y., Cao, X., Jiang, J., and Jia, J. (2007). Fused Costal2 protein complex regulates Hedgehog-induced Smo phosphorylation and cell-surface accumulation. Genes Dev *21*, 1949-1963.

Lu, X., Liu, S., and Kornberg, T. (2006). The C-terminal tail of the Hedgehog receptor Patched regulates both localization and turnover. Genes & development *20*, 2539-2551.

Lum, L., Zhang, C., Oh, S., Mann, R., von Kessler, D., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B., and Beachy, P. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. Molecular cell *12*, 1261-1274.

Marigo, V., Johnson, R.L., Vortkamp, A., and Tabin, C.J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev Biol *180*, 273-283.

McMahon, A., Ingham, P., and Tabin, C. (2003). Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol *53*, 1-114.

Méthot, N., and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell *96*, 819-831.

Méthot, N., and Basler, K. (2000). Suppressor of fused opposes hedgehog signal transduction by impeding nuclear accumulation of the activator form of Cubitus interruptus. Development (Cambridge, England) *127*, 4001-4010.

Méthot, N., and Basler, K. (2001). An absolute requirement for Cubitus interruptus in Hedgehog signaling. Development (Cambridge, England) *128*, 733-742.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science *245*, 371-378.

Monnier, V., Dussillol, F., Alves, G., Lamour-Isnard, C., and Plessis, A. (1998). Suppressor of fused links fused and Cubitus interruptus on the hedgehog signalling pathway. Curr Biol *8*, 583-586.

Motzny, C.K., and Holmgren, R. (1995). The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. Mech Dev *52*, 137-150.

Mukhopadhyay, D., and Riezman, H. (2007). Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science *315*, 201-205.

Müller, B., and Basler, K. (2000). The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic gli-binding sites. Development (Cambridge, England) *127*, 2999-3007.

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J.R., and Ingham, P.W. (1989). A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. Nature *341*, 508-513.

Notredame, C., Higgins, D.G., and Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol *302*, 205-217.

Noureddine, M.A., Donaldson, T.D., Thacker, S.A., and Duronio, R.J. (2002). Drosophila Roc1a encodes a RING-H2 protein with a unique function in processing the Hh signal transducer Ci by the SCF E3 ubiquitin ligase. Dev Cell *2*, 757-770.

Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795-801.

Nybakken, K.E., Turck, C.W., Robbins, D.J., and Bishop, J.M. (2002). Hedgehog-stimulated phosphorylation of the kinesin-related protein Costal2 is mediated by the serine/threonine kinase fused. J Biol Chem *277*, 24638-24647.

Ogden, S., Ascano, M., Stegman, M., Suber, L., Hooper, J., and Robbins, D. (2003). Identification of a functional interaction between the transmembrane protein Smoothened and the kinesin-related protein Costal2. Current biology : CB *13*, 1998-2003.

Orenic, T.V., Slusarski, D.C., Kroll, K.L., and Holmgren, R.A. (1990). Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila. Genes Dev *4*, 1053-1067.

Ou, C.Y., Lin, Y.F., Chen, Y.J., and Chien, C.T. (2002). Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in Drosophila eye development. Genes Dev *16*, 2403-2414.

Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell *78*, 773-785.

Pan, Y., Bai, C.B., Joyner, A.L., and Wang, B. (2006). Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol *26*, 3365-3377.

Pavletich, N., and Pabo, C. (1993). Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. Science (New York, NY) *261*, 1701-1707.

Pepinsky, R., Zeng, C., Wen, D., Rayhorn, P., Baker, D., Williams, K., Bixler, S., Ambrose, C., Garber, E., Miatkowski, K.*, et al.* (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. The Journal of biological chemistry *273*, 14037-14045.

Piwko, W., and Jentsch, S. (2006). Proteasome-mediated protein processing by bidirectional degradation initiated from an internal site. Nat Struct Mol Biol *13*, 691-697.

Porter, J., Ekker, S., Park, W., von Kessler, D., Young, K., Chen, C., Ma, Y., Woods, A., Cotter, R., Koonin, E.*, et al.* (1996). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell *86*, 21-34.

Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., Moses, K., and Beachy, P.A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. Nature *374*, 363-366.

Price, M.A., and Kalderon, D. (1999). Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. Development *126*, 4331-4339.

Price, M.A., and Kalderon, D. (2002). Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1. Cell *108*, 823-835.

Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell *75*, 1401-1416.

Robbins, D.J., Nybakken, K.E., Kobayashi, R., Sisson, J.C., Bishop, J.M., and Therond, P.P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. Cell *90*, 225-234.

Roelink, H., Porter, J.A., Chiang, C., Tanabe, Y., Chang, D.T., Beachy, P.A., and Jessell, T.M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell *81*, 445-455.

Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L., and Therond, P.P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. Nat Cell Biol *5*, 907-913.

Ruppert, J.M., Kinzler, K.W., Wong, A.J., Bigner, S.H., Kao, F.T., Law, M.L., Seuanez, H.N., O'Brien, S.J., and Vogelstein, B. (1988). The GLI-Kruppel family of human genes. Mol Cell Biol *8*, 3104-3113.

Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development (Cambridge, England) *124*, 1313-1322.

Schwartz, C., Locke, J., Nishida, C., and Kornberg, T.B. (1995). Analysis of cubitus interruptus regulation in Drosophila embryos and imaginal disks. Development *121*, 1625-1635.

Schweizer, L., and Basler, K. (1998). Drosophila ciD encodes a hybrid Pangolin/Cubitus interruptus protein that diverts the Wingless into the Hedgehog signaling pathway. Mech Dev *78*, 141-151.

Shin, S.H., Kogerman, P., Lindstrom, E., Toftgard, R., and Biesecker, L.G. (1999). GLI3 mutations in human disorders mimic Drosophila cubitus interruptus protein functions and localization. Proc Natl Acad Sci U S A *96*, 2880-2884.

Sisson, B., Ziegenhorn, S., and Holmgren, R. (2006). Regulation of Ci and Su(fu) nuclear import in Drosophila. Developmental biology *294*, 258-270.

Sisson, J.C., Ho, K.S., Suyama, K., and Scott, M.P. (1997). Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. Cell *90*, 235-245.

Smelkinson, M.G., and Kalderon, D. (2006). Processing of the Drosophila hedgehog signaling effector Ci-155 to the repressor Ci-75 is mediated by direct binding to the SCF component Slimb. Curr Biol *16*, 110-116.

Spemann, H., and Mangold, H. (2001). Induction of embryonic primordia by implantation of organizers from a different species. 1923. Int J Dev Biol *45*, 13-38.

Stegman, M., Vallance, J., Elangovan, G., Sosinski, J., Cheng, Y., and Robbins, D. (2000). Identification of a tetrameric hedgehog signaling complex. The Journal of biological chemistry *275*, 21809-21812.

Sulston, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol *100*, 64-119.

Suzuki, T., and Saigo, K. (2000). Transcriptional regulation of atonal required for Drosophila larval eye development by concerted action of eyes absent, sine oculis and hedgehog signaling independent of fused kinase and cubitus interruptus. Development *127*, 1531-1540.

Tabata, T., Eaton, S., and Kornberg, T. (1992a). The Drosophila hedgehog gene is expressed specifically in posterior compartment cells and is a target of engrailed regulation. Genes & development *6*, 2635-2645.

Tabata, T., Eaton, S., and Kornberg, T.B. (1992b). The Drosophila hedgehog gene is expressed specifically in posterior compartment cells and is a target of engrailed regulation. Genes Dev *6*, 2635-2645.

Taipale, J., and Beachy, P.A. (2001). The Hedgehog and Wnt signalling pathways in cancer. Nature *411*, 349-354.

Taipale, J., Cooper, M., Maiti, T., and Beachy, P. (2002). Patched acts catalytically to suppress the activity of Smoothened. Nature *418*, 892-897.

The, I., Bellaiche, Y., and Perrimon, N. (1999). Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan. Mol Cell *4*, 633-639.

Theodosiou, N.A., Zhang, S., Wang, W.Y., and Xu, T. (1998). slimb coordinates wg and dpp expression in the dorsal-ventral and anterior-posterior axes during limb development. Development *125*, 3411-3416.

Therond, P.P., Knight, J.D., Kornberg, T.B., and Bishop, J.M. (1996). Phosphorylation of the fused protein kinase in response to signaling from hedgehog. Proc Natl Acad Sci U S A *93*, 4224-4228.

Tian, L., Holmgren, R.A., and Matouschek, A. (2005). A conserved processing mechanism regulates the activity of transcription factors Cubitus interruptus and NF-kappaB. Nat Struct Mol Biol *12*, 1045-1053.

Tiniakow, G.G., and Terentieva, E.L. (1933). Cubitus Interruptus, a New Genovariation of the Fourth Chromosome of DROSOPHILA MELANOGASTER. Genetics *18*, 117-120.

Torroja, C., Gorfinkiel, N., and Guerrero, I. (2004). Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. Development (Cambridge, England) *131*, 2395-2408.

Vervoort, M., Crozatier, M., Valle, D., and Vincent, A. (1999). The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the Drosophila wing. Curr Biol *9*, 632-639.

von Mering, C., and Basler, K. (1999). Distinct and regulated activities of human Gli proteins in Drosophila. Curr Biol *9*, 1319-1322.

Von Ohlen, T., and Hooper, J. (1999). The ciD mutation encodes a chimeric protein whose activity is regulated by Wingless signaling. Developmental biology *208*, 147-156.

Von Ohlen, T., Lessing, D., Nusse, R., and Hooper, J. (1997). Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. Proceedings of the National Academy of Sciences of the United States of America *94*, 2404-2409.

Waddington, C.H. (1939). Preliminary Notes on the Development of the Wings in Normal and Mutant Strains of Drosophila. Proc Natl Acad Sci U S A *25*, 299-307.

Wallace, I.M., O'Sullivan, O., Higgins, D.G., and Notredame, C. (2006). M-Coffee: combining multiple sequence alignment methods with T-Coffee. Nucleic acids research *34*, 1692-1699.

Walthall, S.L., Moses, M., and Horabin, J.I. (2007). A large complex containing Patched and Smoothened initiates Hedgehog signaling in Drosophila. J Cell Sci *120*, 826-837.

Wang, B., Fallon, J., and Beachy, P. (2000a). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell *100*, 423-434.

Wang, G., Amanai, K., Wang, B., and Jiang, J. (2000b). Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus. Genes Dev *14*, 2893-2905.

Wang, G., Wang, B., and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus. Genes & development *13*, 2828-2837.

Wang, Q., and Holmgren, R. (2000). Nuclear import of cubitus interruptus is regulated by hedgehog via a mechanism distinct from Ci stabilization and Ci activation. Development (Cambridge, England) *127*, 3131-3139.

Xie, K., and Abbruzzese, J.L. (2003). Developmental biology informs cancer: the emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers. Cancer Cell *4*, 245-247.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. Development *121*, 2265-2278.

Zhang, Q., Zhang, L., Wang, B., Ou, C., Chien, C., and Jiang, J. (2006). A hedgehog-induced BTB protein modulates hedgehog signaling by degrading Ci/Gli transcription factor. Developmental cell *10*, 719-729.

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