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Targets of Cubitus interruptus regulation in the Drosophila embryo

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Targets of Cubitus interruptus regulation in the *Drosophila* embryo

by

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Abstract

The initiation of tissue specific genetic programs requires that a signaling pathway must activate a subset of its complete repertoire of targets at the appropriate time and in the correct location. This is an extreme challenge for a pathway that operates in multiple tissues during the same stage of development. To achieve this level of specificity, tissue specific transcription of target genes relies on the activity of local factors that define a zone of activation competence.

The *Drosophila* Hedgehog (Hh) signaling pathway functions in a variety of tissues, effecting gene activation by modulating the activity of its downstream transcription factor, Cubitus interruptus (Ci). Cells receiving the Hh signal produce a full-length Ci transcriptional activator. Cells that do not receive Hh signal convert full length Ci into a transcriptional repressor. Little concerted effort has been put forth to identify the direct downstream targets of Ci that mediate the effect of Hh signaling. In this work, I use a combination of genomic approaches to identify regions in the genome where Ci binds (DAMID) and to determine the genes that respond to Hh signaling (expression array analysis) in the *Drosophila* embryo.

I find that DAMID signals for repressor (DamCiRep) and activator (DamCiAct) forms of Ci overlap hundreds of times indicating that repressor and activator forms of Ci recognize the same sequences *in vivo*. Transcriptional profiling of Hh pathway mutants uncovers genes that respond to all genetic backgrounds as well as sub-clusters of genes

that change expression levels in specific mutant backgrounds. High confidence putative Ci targets are found to be largely tissue specific in their expression and function. A validation of putative Ci targets via in situ hybridization reveals a tissue specific response to Ci in three developmental systems: the embryonic visual system, dorsal ectoderm, and the developing tracheal system. Sequence specific binding sites for tissue specific regulators are present in Ci enhancers leading to a model by which Ci functions in synergy with local factors for optimal gene activation.

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Chapter 1

Introduction

Developmental biology is the study of the process by which a single fertilized cell is transformed into a multi-cellular organism. This transformation requires cell growth and division, axis specification, differentiation, cell-fate specification, morphogenesis, programmed cell death, and pattern formation. A central theme in developmental biology is how groups of cells in a growing organism become different from their neighbors. During oogenesis of the fruit fly *Drosophila melanogaster*, the basic framework on which all patterning events in the fly's life depends is provided by a maternal contribution that specifies positional information along the dorso-ventral and anterior-posterior axes. With a pre-pattern in place, cellularization and the onset of zygotic gene activity must guide subsequent cell fate decisions that determine all aspects of the organism's development. Although axis determination differs from flies to vertebrates, the basic question regarding the development of distinct tissue types from an undetermined group of cells is the same for all animals: How do cells communicate with one another?

Early developmental biologists conducted experiments aimed at answering whether cell-cell communication was required or whether different regions of the embryo developed independently of each other. Spemann and Mangold dramatically demonstrated the principle of induction in amphibians, whereby a signal from one group of cells influenced the development of another. By grafting the dorsal lip of a gastrulating

embryo to a host gastrula they induced a new body axis containing a neural tube and somites (Spemann and Mangold, 2001). Spemann and Mangold applied the term "organizer" to the grafted tissue because it harbored special properties capable of organizing surrounding cells into specific tissue types. We now understand that the basis for that transformation is partly due to the secretion of neuralizing factors that are specific to the organizer such as noggin and follistatin ((Lamb et al., 1993), (Hemmati-Brivanlou et al., 1994).

Inductive signals that move out from one tissue to cause developmental changes in another is a common theme in developmental biology. A major step forward in the identification of many inductive signals and their transduction machinery have come from mutagenesis screens that reveal visible pattern defects in the body plan of model organisms. For example, Christiane Nüsslein-Volhard and Eric Weischaus screened for genetic mutations that altered the denticle pattern of the *Drosophila* larvae cuticle (1980). Subsequent mapping and cloning of the genes uncovered by their screen has led to a detailed understanding of many patterning mechanisms in the fruit fly that are also conserved across species. One such protein, encoded by the *Drosophila hedgehog* (*hh*) gene, is expressed in the posterior compartment of each embryonic segment (Tabata et al.,1992) and imaginal disc (Lee et al., 1994, Tabata et al., 1994). Hh is an example of a secreted protein that confers special properties to cells by altering gene expression.

Hedgehog as a developmental organizer

The earliest indication of Hh-dependent inductive properties came from *hh* mutations that revealed loss of specific cell fates in each segment of the *Drosophila* embryo resulting in a disrupted anterior/posterior polarity (Nusslein-Volhard and Wieschaus, 1980)The role of *hh* as a segment polarity gene relies on activation by the Engrailed (En) transcription factor in the posterior compartment of each segment (Tabata et al., 1992). Hh protein initially stabilizes the expression of *wingless* (*wg*) in anterior cells, another secreted factor which feeds back in a positive regulatory loop to activate *en* in posterior cells (DiNardo et al., 1988). Hh and Wg signals become independent of each other by embryonic stage 11 and function as a bi-partite organizer, controlling cell-fate decisions by inducing short-range signals in opposite directions along the A/P axis of the embryo (Alexandre et al., 1999). Wg signals in the anterior direction, specifying naked cuticle fates by repressing *shavenbaby* (Payre et al., 1999) as well as *rhomboid* (*rho*) and Serrat*e* (Ser) expression (Alexandre et al., 1999). Hh affects patterning in the posterior direction (the most anterior cells of the adjacent segment) by activating *rho* and suppressing Ser. The effect of activating these target genes is to induce the larval cells to secrete cuticle with specialized structures called denticles. Hh and Wg signals are mutually antagonistic by stage 11and signal uni-directionally (Gritzan et al., 1999)Interestingly, Hh signaling is capable of activating the target gene *patched* (*ptc*) in both directions (Ingham et al., 1991). Repression of the Hh target gene *stripe* anterior to the *hh* expression domain requires a *cis*-regulatory element that interacts with the sequence specific binding factor of the Wg signaling pathway, Pangolin (Hatini and

DiNardo, 2001). This result implies that the mechanism of the mutually antagonistic Wg and Hh signals lies at the transcriptional level of individual target genes.

Perhaps the best studied example of Hh organizing function is in the *Drosophila* wing imaginal disc. Analysis of Hh expression in the wing disc led to the hypothesis that compartmentalized *hh* was important for organizing the development of tissues in a non-autonomous way (Tabata and Kornberg, 1994). *hh* expression is activated in posterior cells by *en* (Tabata et al., 1992; Zecca et al., 1995) and Hh protein is secreted into the anterior compartment where it accumulates at the A/P compartment border and activates the expression of *patched* (*ptc*) (Tabata and Kornberg, 1994) and *decapentaplegic* (*dpp*) (Zecca et al., 1995). The targets of Hh signaling exhibit a partially overlapping distribution in anterior cells of the wing disc, arguing that proper patterning of the wing relies on a Hh activity gradient (Strigini and Cohen, 1997).

The inductive properties of Hh are limited to the anterior cells in which it accumulates, an important feature of the Hh organizer. Engrailed (En) expression in posterior cells negatively regulates the expression of the terminal transcriptional mediator of the Hh signaling pathway, Cubitus interruptus (Ci) (Eaton and Kornberg, 1990) and therefore, Hh target genes are not activated in posterior cells. *en* deficient clones in the wing imaginal disc result in new interfaces of *en* positive and *en* negative cells causing pattering defects consistent with ectopic organizers (Tabata et al., 1995). Thus, compartmentalization of Hh activity in the anterior by En in is an important feature of the

Hh developmental organizer that ensures the powerful, inductive properties of Hh are limited to a restricted group of cells.

Regulation of Cubitus interruptus by Hedghog signaling

Ci was originally identified by Nusslein-Volhard and Weichhaus in their mutagenesis screen for genes involved in pattern formation (Nusslein-Volhard and Wieschaus, 1980) and belongs to the Gli family of transcription factors that share a conserved zinc finger DNA binding domain (Matise and Joyner, 1999). Hh induced gene activation requires the activity of Ci(Méthot and Basler, 2001), believed to be the only transcription factor directly mediating the Hh signal in *Drosophila*. The vertebrate homologs of Ci (Gli1, 2, and 3) regulate gene transcription via repression and activation capabilities. Gli1 functions as a transcriptional activator (Ruiz i Altaba, 1998), Gli2 is primarily an activator (Aza-Blanc et al., 2000), and Gli3 functions as a repressor (Wang et al., 2000).

In flies, the formation of a Ci transcriptional activator is the result of an intracellular signaling cascade that starts with the binding of Hh at the cell membrane to its putative receptor Ptc (Chen and Struhl, 1996; Ingham et al., 1991; Lu et al., 2006), a twelve-pass membrane protein with similarities to channel proteins and transporters (Hooper and Scott, 1989; Nakano et al., 1989). Exposure of cells to Hh inhibits processing of the full length 155 kilodalton (kD) Ci protein (Ci-155) to a truncated 76kD

repressor form (Ci-76) (Aza-Blanc et al., 1997) and converts Ci-155 to a transcriptional activator (Méthot and Basler, 1999). Hh association with Ptc inhibits negative regulation on Smoothened (Smo) (Alcedo et al., 1996) (Taipale et al., 2002) a seven-pass membrane protein with homology to G-protein coupled receptors. Hh binding leads to extensive phosphorylation of the cytoplasmic tail of Smo by protein kinase A (PKA) and casein kinase 1 (CK1) leading to its subsequent stabilization at the cell surface and activation (Denef et al., 2000). The mechanism by which Ptc negatively regulates Smo is unknown. Recent evidence suggests that Smo activation relies on the neutralization of Arg clusters in the C-terminal tail by phosphorylation, causing a conformational change in the protein (Zhao et al., 2007). The phosphorylation state of Smo acts as conformational switch, preventing its association with factors that negatively regulate the pathway (Zhao et al., 2007).

The C-terminal tail of Smo binds directly to Cos2, a kinesin-like protein that acts as a scaffolding molecule, bringing together cytoplasmic components of the pathway that inhibit pathway activity (Lum et al., 2003); (Jia et al., 2003). These include Ci-155, the Ser/Thr kinases Fused (Fu), Protein kinase A (PKA), casein kinase I (CKI), and glycogen synthase kinase 3 (GSK3) and forma complex that is required for efficient processing of Ci-155 to Ci76 (Chen and Struhl, 1998); (Jia et al., 2002); (Price and Kalderon, 2002)The presence of Hh signal allows for phosphorylation of Cos2 and partial disassociation of the Cos2, Fu, Ci-155 complex (Jia et al., 2003); (Ruel et al., 2003);(Ruel et al., 2007), leading to stabilization and activation of Ci-155.

Regulation of Hh signaling targets by Ci/Gli

A major question regarding the output of the Hh signaling pathway is how a variety of transcriptional effects can be elicited by a single transcription factor. In the wing disc, Hh signaling activates the targets *dpp* and *ptc* in thin stripes at the A/P border, yet the expression domains of *dpp* and *ptc* are not completely coincident. *ptc* expression is nested within the larger domain of *dpp* expression. This raises the possibility that individual targets respond to different threshold levels of Hh signal, which is supported by experiments utilizing temperature sensitive alleles of Hh. Expression of a high-level gene such as *en* is lost before expression of a low-level gene *ptc* as temperature is increased (Strigini and Cohen, 1997). In addition, loss of *ptc* in clones which alleviate repression of the Hh signaling pathway, have differential effects on target gene expression depending on where the clone develops in the anterior compartment of the wing disc. Low-level genes would activate in *ptc* clones anywhere in the anterior compartment, while high level genes would only activate in clones near the A/P border (Strigini and Cohen, 1997). This suggests that *ptc* mutant clones remain sensitive to Hh activity and that additional Hh activity is required to activate high-level genes even though the pathway has been partially de-repressed.

Ci is a requirement for Hh induced gene expression (Méthot and Basler, 2001)and therefore must be able to detect differences in Hh signaling. A mechanism for interpretation of the short range Hh activity gradient in the wing disc has been suggested,

taking into account the bi-functional nature of the Ci transcription factor (Hooper and Scott, 2005). In the absence of Hh signal, for example in the far anterior of the wing disc, full length Ci is converted to repressor which deactivates target genes. Low levels of Hh are capable of blocking this processing, leading to de-repression of some target genes and not others. A third interpretation of the gradient occurs when high levels of Hh prevent Ci repressor from forming and convert full length Ci into an activator, thus stimulating the expression of high level target genes. This model is consistent with the idea that different classes of genes could be responding to relative ratios of Ci activator and Ci repressor. Ci loss of function clones in the far anterior of the wing disc result in ectopic expression of *dpp* suggesting that loss of Ci and thus, loss of Ci repressor activity leads to de-repression. At the border, however, *dpp* expression is not affected by ectopic Ci repressor, suggesting that in this situation *dpp* expression also requires Ci activator function (Méthot and Basler, 1999). This model is further supported by data showing that Ci activator and Ci repressor both function at common binding sites at individual promoters (Müller and Basler, 2000).

The Hh signaling gradient and the model that predicts positive and negative activities of Ci as a means to interpret the gradient is further supported by experiments in vertebrates. The vertebrate neural tube is a system with striking parallels to the wing disc. Sonic hedgehog (Shh), the vertebrate homolog of Hh, is responsible for inducing long-range pattern by differential gene expression from a localized source. As Shh is secreted dorsally from its ventral-most position, different cell fates are specified along the D/V axis. Mutants that lack all Shh activity fail to differentiate the 6 types of neurons that are

specific to the ventral half of the spinal cord (Wijgerde et al., 2002). Genes activated by Shh are distinguished as Class II transcription factors. For example, ClassII genes *Nkx2.2* and *Nkx6.1* are activated by peak levels of Hh signaling in tissue immediately adjacent to *Shh* expression in the floor plate. Class I genes (Shh repressed) are expressed more dorsally, further from the Shh source and together, the differential expression of Class I and Class II genes and their subsequent activities subdivides the neural tube into 5 distinct domains (Briscoe et al., 2000).

The organization of the ventral neural tube into neuronal subtypes appears to be carried out by an interpretation of the Shh gradient by the Gli transcription factors. For example, peak levels of Sonic are required to prevent Gli2 processing and to preserve its activator activity (Aza-Blanc et al., 2000) while Gli3 functions as a repressor (Wang et al., 2000). Gli2 -/- embryos exhibit a loss of floor plate differentiation concomitant with loss of targets that respond to peak levels of Sonic (Ding et al., 1998); (Matise et al., 1998) Neural tubes that are mutant for the Gli3 repressor are indistinguishable from wildtype with respect to floor plate gene expression. However, markers of intermediate ventral nerve cord are expanded in Gli3 mutants at the expense of more dorsally located markers (Persson et al., 2002). In embryos lacking all Gli activity, ventral cell types are missing and the spatially segregated expression patterns of target genes is severely disrupted, as seen in Shh mutants (Bai et al., 2004). Taken together, these results indicate that the Shh gradient is interpreted by the Gli proteins to ensure proper gene expression and patterning.

Tissue specific response to Hedgehog

Although it is clear that the Ci/Gli proteins are responsible for Hh induced pattering, it is not apparent how they activate subsets of Hh targets in specific tissues. In the *Drosophila* embryo, Hh operates at multiple sites, activating gene expression that is required in certain tissues but not in others. As discussed above, Hh induces *wingless* in the embryonic ectoderm to establish an organizing center that patterns each segment. However, Hh does not induce *wg* in the wing disc, where Wg is required for proper patterning along the D/V axis. Also, Hh does not induce *rhomboid* expression in the wing disc, which is required for localized activation of the EGF receptor pathway. Other examples of tissue specific gene induction by Hh include the activation of *lethal of scute* (*l'sc*) in the anterior midline daughter cells. This event leads to the specification of ventral unpaired median neurons and the median neuroblast (Bossing and Brand, 2006). A requirement for Hh in the development of specific neuroblast lineages has also been reported (McDonald and Doe, 1997). McDonald and Doe show that *huckebein* expression in rows 1/2 of the neuroectoderm depends on Hh activity, consistent with Hh expression in the neuroectoderm (Tabata and Kornberg, 1994).

In many instances, a correlation can be drawn between the expression of a tissue specific gene and the domain of Hh signaling. Such is the case for the homeobox gene *bagpipe* (*bap*) in the developing visceral mesoderm. The anterior edge of *bap* expression

perfectly abuts the parasegment border as marked by *engrailed* in the overlying ectoderm. *bap* expression is severely reduced in Hh mutant embryos while it is greatly expanded in *wg* mutants (Azpiazu et al., 1996). It is likely that the induction of *bap* by Hh in the visceral mesoderm is due to secreted ectodermal Hh since ectopic expression of Hh in the ectoderm has the same effect on visceral mesoderm markers as does Hh expression driven specifically in the mesoderm (Bilder and Scott, 1998). Given the diverse number of roles that Hh plays in tissue specific development, a major unresolved question is the mechanism that allows for tissue specific gene activation by Ci.

Signaling and Ci specificity

The issue of signaling specificity has been a topic of interest in developmental biology for the last thirty years. In animals, the vast majority of developmental processes are controlled by seven major cell-cell signaling pathways: Wnt, TGF-β, Hedgehog, receptor tyrosine kinase (RTK), nuclear receptor, Jak/STAT, and Notch (Gerhart, 1999). Even though each activated pathway has a unique mechanism for transducing signal, the endpoint is always to activate or repress gene expression. Since each pathway is used repeatedly during development, activation or repression of subsets of a given pathway's genetic targets must be successfully accomplished in a variety of tissues and developmental contexts. How subsets of targets are activated in specific tissues by any given signaling pathway is a matter of great interest.

The study of nuclear receptors (NRs) and their effects on transcriptional regulation has provided tremendous insight into the mechanisms underlying gene specific transcriptional control. Nuclear receptors are transcription factors that exert their effects on gene transcription by directly binding to response elements in DNA, typically upstream of the core promoter of target genes. Work using purified receptor fractions led to the model that ligand binding to receptors caused receptor dimerization and an increased affinity for cis-acting DNA regions termed hormone response elements (or HREs) near target genes (Payvar et al., 1982). Steroid hormones elicit a variety of physiolocigal effects such as, but not limited to: sex organ development, bone homeostasis, stress response, and regulation of the menstrual cycle. How a single hormone can affect different tissues has prompted researchers to address the ability of individual steroid hormone receptors to elicit a variety of transcriptional responses. Using naturally occurring and synthetic reporter genes for the retinoic acid receptor (RAR) Nagpal et al. showed that the response to retinoic acid at the transcriptional level varied depending on the nature of the RAR isoform or the cellular context of the promoter (Nagpal et al., 1992), building a strong circumstantial case for the involvement of co-activators at the promoter level. Indeed, a seminal screen for hormone receptor co-factors using the yeast two-hybrid approach led to the discovery and cloning of many members of the SRC/p160 family of ligand induced co-factors (Lee et al., 1995). This allowed *in vivo* testing to determine physiological relevance of co-factors. For example, gene targeted knockout of the putative nuclear receptor co-factor SRC-1 resulted in mice that were partially resistant to the effects of steroid hormones (Xu et al., 1998). However, as examples of NR/co-factor complexes are being identified for gene-specific regulation

(Ito et al., 2000), many co-factors are ubiquitously expressed and therefore, the regulatory code for optimal transcriptional activation and specificity by NRs is still being determined on a gene by gene basis.

As new studies continue to reveal the complexity of transcriptional activation by ligand induced signaling pathways, it is increasingly clear that activation of a given pathway is not sufficient to activate gene expression in a context dependent manner. Instead, tissue specific regulators converge on cis-regulatory element(s) along with signal activated transcription factors to trigger a specific response. The mesodermal *Drosophila even-skipped* enhancer is an excellent example of this type of regulation. *eve* is expressed in a small subset of somatic mesodermal cells that are progenitors for specific muscle fiber and heart accessory cells. The progressive development of the *eve* expressing progenitors requires a pre-patterning step by the *wg* and *dpp* pathways that facilitates the induction and response to Ras/RTK signaling (Halfon et al., 2000). Cloning of the *eve* Muscle and Heart enhancer (MHE) and fusion to LacZ as a transgenic reporter revealed a response to Dpp, Wg, and RTK/Ras signaling that exactly phenocopied the response of endogenous *eve*. Among the various binding sites for transcription factors in the MHE that mediate Dpp, Wg, and RTK/Ras signaling were binding sites for the mesodermal specific regulators Tinman and Twist. In cells in which the three pathways are active, the MHE enhancer is silent unless also bound by Tinman and Twist, revealing a tissue specific input for *eve* expression (Halfon et al., 2000). The stark principle of "all or none" activation exemplified by the *eve* MHE is a repeating theme for other signaling pathways, particularly of Notch signaling, where enhancers

may contain multiple high-affinity binding sites but are only activated in one distinct tissue throughout the life of the fly (Barolo and Posakony, 2002). However, tissue specific factors may also contribute to activation synergy by recruiting ubiquitous proteins such as chromatin remodeling complexes or components of the basal transcription machinery.

As I have mentioned in previous sections of this introduction, the Hh signaling pathway has been shown to be essential for several tissue-specific developmental processes. Still, many of the phenotypes associated with mutations in the Hh gene (Flybase, 2009) are orphan phenotypes in the sense that the genes downstream of Hh responsible for the normal development of those tissues are unknown. Moreover, the mechanisms by which Ci exerts tissue-specific gene activation remain largely undiscovered. Evidence for Ci as a necessary but not sufficient transactivator of gene expression does exist, however, in a study of the *dpp* "heldout" (*dppho*) enhancer in the wing disc (Hepker et al., 1999). Dpp is lost in *ci* loss of function clones and the *dppho* enhancer, a cis-regulatory element containing Ci binding sites appears to recapitulate the endogenous *dpp* pattern when cloned and fused to LacZ. However, *dppho-LacZ* expression is restricted to certain areas when the wing disc is challenged with over expression of Ci. *dppho-LacZ* exhibits a different pattern of ectopic expression in response to either activated Wg signaling or expression of Vestigial, a candidate wing selector gene required for wing specific gene expression (Halder et al., 1998). Consistent with these findings are the presence of Pangolin transcription factor binding (for Wg signaling) and Vestigial binding sites in *dppho*. Lastly, the authors found that *LacZ*

expression is not induced in the full *dppho* pattern when fused to four Ci consensus binding sites, arguing for the activity of other tissue specific regulators.

A general determinant in Ci mediated gene activation is Creb binding protein or CBP. Mutations in CBP lead to loss of *en* and *wg* expression in the embryo as well as loss of *ptc* expression in the wing disc. It was shown that CBP binds to a region within the Ci protein previously identified as a Ci activation domain (Alexandre et al., 1996) and increases Ci activity in a dose dependent way (Akimaru et al., 1997).

In mammals, recent evidence is becoming available to support the idea that Glis function in concert with other factors for positive regulation on *cis*-regulatory elements. Chromatin immunoprecipitation (ChIP) has been successfully used to identify targets of Gli1 during the course of Shh mediated neural patterning (Vokes et al., 2007). Moreover, their analysis of Gli dependent, enhancer driven expression revealed the need for tissue specific regulators other than Gli to recapitulate the wildtype pattern.

In this study, I have taken a genomic approach to uncover novel gene targets that function downstream of Hh signaling. Using the DNA Adenine Methyltransferase Identification technique (DAMID), I mapped the binding regions of both a Ci Activator (DAMCiAct) and Ci repressor (DAMCiRep) in the context of the stage 10-11 Drosophila embryo. I found that DAMID signals for repressor and activator forms of Ci significantly overlap hundreds of times at known and novel targets, substantiating previous results and providing evidence that both forms recognize the same sequences on a global scale. Most of the putative Ci targets associated with DAMID signal show tissue specific expression

suggesting that Hh functions on a local rather than universal scale. To corroborate the DAMID results, I performed transcriptional profiling of Hh pathway mutants and uncovered genes that respond to all mutants tested as well as sub-clusters of genes that specifically change expression levels in some backgrounds but not others, arguing for a non-linear distribution of Hh signaling targets. Finally, I validated putative Ci targets by expressing Ci activator ubiquitously in the embryo and assayed the response via *in situ* hybridization. This study reveals a tissue specific response to Ci activator in three developmental systems: the embryonic visual system, the dorsal ectoderm, and the developing tracheal system. In addition, I found that *ci* expression is itself modulated by Hh signaling, perhaps revealing a conserved positive regulatory loop between Shh/Hh and Gli1/Ci. I also discovered that sequence specific binding sites for local regulators are present in Ci enhancers leading to a model by which Ci functions in synergy with local factors for optimal gene activation.

Chapter 2

Identification of Ci targets

Introduction

The Hedgehog (Hh) signaling pathway functions by inducing gene expression to control various aspects of development. Two genes appear to be activated as a general response: *patched* (*ptc*) (Hidalgo and Ingham, 1990; Tabata et al., 1994) and *roadkill* (*rdx*) (Kent et al., 1996; Zhang et al., 2006) and function in negative feedback loops to attenuate Hh signaling. Induction of other Hh targets appears to be determined by the context of the tissue in question. *Drosophila* has yielded several examples of tissue-specific, Hh induced gene activation. Patterning of each segment in the embryonic ectoderm requires the induction of *wg* expression by Hh in anterior compartment cells (Ingham and Hidalgo, 1993). After Hh and Wg signaling domains are established, Hh signals uni-directionally to simultaneously activate and repress gene expression (Gritzan et al., 1999; Alexandre et al., 1999). Similarly, separation of mesoderm into Anterior and Posterior domains requires the induction of *bagpipe*, most likely by secreted Hh from the overlying ectoderm (Azpiazu et al., 1996). The wing imaginal disc is also a well-studied model of Hh signal transduction and target activation. Hh induces *ptc* and *dpp* in anterior cells at the A/P compartment border in partially overlapping territories (Tabata and Kornberg, 1994; Strigini and Cohen, 1997). All the effects of Hh signaling seem to be mediated by Cubitus Interruptus (Ci) (Methot and Basler, 2001), a zinc-finger

transcription factor with extensive homology to the vertebrate Gli proteins. Hh induced gene activation is carried out by modulating the activity of Ci; cells which receive the Hh signal convert full length Ci into a transcriptional activator, thereby preventing Ci processing into a transcriptional repressor (Aza-Blanc et al., 1997; Methot and Basler, 1999).

The search for components of the Hh signaling pathway as well as its targets has been an intense field of study, employing the use of genetic, biochemical, and genomic screens. For the purpose of identifying Hh targets, arrays have been used to probe cell lines with stable Gli1 expression (Yoon et al., 2002). Arrays have also been used to ask how Hh controls a known process such as the proliferation of a neuronal sub-type in the brain (Oliver et al., 2003). While studies of converging factors at single enhancers to demonstrate a tissue specific regulatory "code" for Ci/Gli are rare (Hepker 1999), new evidence is becoming available to support the idea that Ci/Gli function in concert with other factors at *cis*-regulatory elements for gene activation. The advent of techniques capable of identifying regions in the genome that interact with specific transcription factors have shed light on potential direct targets of Ci/Gli. Chromatin immuno-precipitation (ChIP) has been successfully used to identify targets of Gli1 during the course of Shh mediated neural patterning (Vokes et al., 2007). Moreover, their analysis of Gli dependent, enhancer driven expression revealed the need for tissue specific regulators other than Gli to recapitulate wildtype expression pattern. Despite these advances, the number of identified direct targets of Ci is small. Furthermore, the

mechanisms by which Ci imposes positive and negative regulation on its targets is not understood.

In this study, I use Ci directed DNA Adenine Methyltransferase (DAMID) to show that Ci activator and Ci repressor interact with genome at hundreds of overlapping locations. In addition, I employed an expression array analysis to identify genes that respond to Hh signaling. The combined genomic approaches reveal a high correspondence between regions where Ci is bound and genes that respond to Hh signaling. Transcriptional profiling of Hh pathway mutants uncovers genes that respond to all mutants as well as sub-clusters of genes that specifically change expression levels in some backgrounds but not others. I established that most of the putative Hh targets are expressed or function in a tissue specific manner. To validate novel Hh targets and to test the extent to which CiAct can function in activating gene expression, I expressed CiAct ubiquitously. I outline a tissue-specific response in three developmental systems: the embryonic visual system, dorsal ectoderm, and the developing tracheal system. In addition, I find that *ci* expression is itself modulated by Hh signaling. Sequence specific binding sites for tissue specific regulators are present in Ci enhancers leading to a model by which Ci functions in synergy with local factors for optimal gene activation.

Results

Identifying Ci binding regions in the Drosophila genome

To identify Ci binding regions in the *Drosophila* genome I utilized the DamID technique, an established method for identifying transcription factor binding regions *in vivo*. DamID utilizes DNA methylase activity of an engineered fusion protein composed of DNA adenine methyltansferase (DAM) and the DNA binding domain of a transcription factor of interest (van Steensel et al., 2001). Specific binding by the transcription factor directs methylation of linked sequences. Ci exists as two forms, a transcriptional activator that is generated upon Hh signal transduction, and a repressor form that is processed from the full-length protein in tissues where *hh* signaling is not active (Aza-Blanc et al., 1997). To identify genomic regions that are bound by Ci, I generated a DAM Ci Activator (DAMCiAct) construct by fusing DAM to the N-terminus of Ci lacking consensus protein kinase A (Pka) phosphorylation sites (Cim1-m4, Chen 1999). This constitutively active form of Ci has been shown to activate Hh target genes independently of Hh signaling. To identify genomic regions bound by Ci repressor, I fused DAM to the N terminus of Ci76 (Aza-Blanc, 1999) to generate DAMCiRep. Both constructs were placed downstream of a UAS-regulated minimal promoter and transgenic fly lines were generated. Activity of the Ci activator and Ci repressor fusion contructs were assessed in the wings of animals carrying the MS1096 GAL4 enhancer trap, which expresses strongly in the wing disc. Wing phenotypes indicated activator and repressor activity in the respective lines (data not shown).

To identify Ci targets in the embryo and to reduce non-specific methylation, DAMCiAct and DAMCiRep expression was examined in animals that did not carry a GAL4 enhancer transgene. Expression of these "un-induced" fusion constructs was determined to result in sufficient levels of methylation, in contrast to GAL4-driven expression which resulted in lethality or non-specific methylation. A third fly line, one that carries the DAM alone served as a control for non-specific methylation.

Methylated DNA fragments from experimental and control samples were recovered from stage 10-11 embryos and the fragments were PCR amplified to generate suitable quantities for hybridization to whole genome tiling arrays. Hybridized arrays were scanned and fluorescent intensity ratios were calculated for three independent biological replicates. To identify statistically relevant binding regions, an analysis was performed to calculate a p-value for each log2 transformed ratio. Next, a sliding window (N=4) scanned consecutive probes for significant averaged DAMCi/DAM log ratios. In this way, features on the array representing neighboring sequences on the chromosome were evaluated based on their significance score and identified as high probability binding regions for Ci if they met or exceeded that score (Analysis developed by Katerina Kechris, see experimental procedures for details).

Overlapping DAMCiAct and DAMCiRep binding regions

A previous report showed that CiAct and CiRep are capable of functioning through a common Gli consensus sequence (Müller and Basler, 2000). Genomic positions of DAMCiAct and DAMCiRep binding regions were compared to identify the

number of times they overlapped by at least 1 base pair. A summary of the results from the DAMID experiments is given in Table 1. A statistical analysis reveals that a majority (51%) of peaks from the DAMCiRep experiment overlap with those of the DamCiAct experiment (K. Kechris, methods). This suggests that both DAMCi fusion proteins are capable of recognizing the same sequences *in vivo*. Another representation of the overlap between experiments is the tally of nearest upstream and downstream genes relative to each binding region that are shared between the two experiments. The two DamID experiments identified a total of 1825 shared genes, representing approximately 66% (1825/2747) of the genes near DamCiRep peaks and approximately 52% (1825/3521) of the genes near DamCiAct peaks.

Enrichment of Ci consensus binding motif within DAMID binding regions

As a further validation of the DamID technique, I sought to determine the enrichment of Ci consensus binding motifs within the DAMID binding regions. The consensus binding site for Gli/Ci is TGGGTGGTC (Kinzler and Vogelstein, 1990; Pavletich and Pabo, 1993) and although the consensus site seems to be preferred (Hallikas, 2006), Ci has been shown to regulate gene transcription through degenerate sites as well (Hepker et al., 1999). I searched the *Drosophila* genome for the consensus Ci motif and calculated its enrichment in DAMID binding regions. Based on its frequency of occurrence in the genome relative to its occurrence in binding regions for either DAMCiAct or DAMCiRep (experimental procedures), I find that the Ci consensus motif is enriched ~2.4 fold in DamCiRep and ~2.5 fold in DamCiAct binding regions (K. Kechris, methods, Table 1).

DamID binding regions at known Hh target loci

As proof of principle, I examined the DAMID signal at loci of *hh* signaling targets that are known to be directly regulated by Ci or require *hh* for their expression. I plotted the statistically significant log2 transformed DamCi/Dam ratios for both DAMCiAct and DAMCiRep against their genomic coordinates at the loci of four known Hh targets (Figure 1). In the context of the embryo, sequence specific binding regions for Ci have been identified that regulate the expression of *patched* (*ptc*) (Forbes et al., 1993; Alexandre et al., 1996) and *wingless* (*wg*) (Von ohlen 1997; Lessing and Nusse 1998). Significant DAMCiAct signal spans approximately 30kb, covering most of the *ptc* transcription unit and ~17kb of upstream sequence (Figure 1A, orange bars). This signal overlaps with a fragment approximately 700 base pairs upstream of the *ptc* transcription start site that contains a cluster of 3 Ci consensus binding sequences Figure 1A, red square). Interestingly, DamCiRep binds to sequences within the first intron and ~7kb upstream, not overlapping with previously identified Ci motifs. Lessing and Nusse (1998) identified a 4.5 kb sequence upstream of *wg* that contained elements that drove *wg*-like stripes and conferred negative regulation on *wg* expression. Significant signal for both DAMCiAct and DAMCiRep encompass this fragment (Figure 1B).

roadkill (*rdx*) and *huckebein* (*hkb*) are two genes that have been reported to require Hh activity for their expression (Kent et al., 2006; McDonald et al.,1997). The DAMCi experiments revealed binding within the *rdx* transcription unit with overlapping

signal for both experiments (Figure 1D) suggesting CiAct and CiRep share common regulatory elements. The majority of DAMID signal at the *hkb* locus consists of 4 significant overlapping binding regions for both DAMID experiments (Figure 1C). In addition, we analyzed the loci of nine other genes known to be dependent on *hh* signaling in the embryo: *wingless* (Ingham and Hidalgo, 1993), *engrailed* (Bossing et al., 2006), *rhomboid* (Alexandre et al.,1999), *DWnt-4* (Buratovitch et al., 2000), *Drop*(D'Alessio et al., 1996), *lethal of scute* (Bossing et al., 2006), *seven up* (Ponzielli et al., 2002), *ladybird early* (Jagla et al., 1997), and *bagpipe* (Azpiazu et al., 1996). In all cases, DAMCiAct and DAMCiRep overlapping peaks were found in or within 1kb of the transcription unit (data not shown), consistent with the idea that these genes are direct targets of Ci and my application of the DAMID technique identified biologically relevant binding regions.

Ranking of overlapping DAMID regions

729 DAMCiAct binding regions shared common sequences with DAMCiRep binding regions. I created a significance ranking of the 729 DAMCiAct binding regions based on p-value and found that the above-mentioned known *hh* signaling targets are associated with some of the most significant binding regions (13^{th}, 43^{rd}, 59^{th} and 63^{rd} for *l'sc*, *ptc*, *en* and *wg*), thus correlating overlap between the two DAMID experiments with known targets of Hh signaling. Not all putative Hh targets were present in the group of genes associated with overlapping binding regions. For example, *Serrate*, a target of negative regulation by Hh signaling in the embryo (Alexandre et al., 1999) was not associated with overlapping DamCiAct and Dam CiRep binding regions and had only a relatively weak DamCiAct signal (not shown).

DAMCi experiments reveal tissue specific targets

By visual inspection, I identified 351 genes that were associated with high-ranking DAMCiAct binding regions. Based on expression pattern or mutant phenotype, 147 of these genes can be classified by tissue type in which they are expressed or function (Flybase, Figure 2). The most prevalent categories are nervous system, tracheal, and mesoderm or mesodermal derivatives such as heart (Figures 3, 4, and 5).

The identification of mesoderm expressing genes by the DAMCi experiments is consistent with the fact that specification of the visceral mesoderm requires the inductive Hh signal for subdivision of the mesodermal parasegments (Azpiazu et al., 1996; Hosono et al., 2003). In Figure 3, I provide several examples of visceral mesoderm markers that are expressed in the characteristic broken line pattern of the visceral mesoderm. In addition, I identified several genes that are expressed in heart precursor cells (Figure 3 arrowheads). A direct role has been proposed for Hh in inducing *eve* positive progenitors along the cardiac mesoderm (Liu et al., 2006).

Genes involved in nervous system development comprise the largest group of tissue-specific genes identified by the DAMID experiments. Of particular interest is the sub-group of genes expressed in neuroblasts, a self-renewing population of cells in the neuroectoderm that give rise to terminally differentiating neurons. Matsuzaki and Saigo (1996) produced an elegant map of Hh associated defects in the neuroblast pattern. I identified several genes that appear to be expressed in all neuroblasts (Figure 4,

CG12708, *miranda*, CycE, and *string*) or subsets of neuroblasts (*PDM2*, *hunchback*, *huckebein*, and *elav*). Consistent with these results, McDonald et al (McDonald et al., 1997) showed that *hkb* expression is directly dependent on Hh signaling in neuroblast rows 1/2 and 7 .

hedgehog was first described as a gene that affects segmentation and the alternating pattern of naked versus denticle covered cuticle in the embryonic epidermis. We now know that those patterning effects are mediated by Hh induced gene expression in the ectoderm (see introduction). The DAMID experiments uncovered many genes that are expressed in patterns that are consistent with Hh dependent induction in the epidermis. Among them, I have determined that *Tom*, *ImpL2*, *Amalgam*, *derailed*, and *18 wheeler* are expressed in stripes corresponding to the Hh signaling domain by either the segment groove morphological marker (Figure 5, *Tom* arrow) or by genetic markers such as Hh lacZ or Engrailed (not shown). In addition, several gene expression patterns mark the invaginating ectoderm that forms the tracheal pits at the onset of tracheal development (Figure 5 arrowheads). Hh has been shown to play a role in patterning the primary outgrowth of the tracheal branches from these structures (Glazer and Shilo, 2001). However, the genes identified by DAMID exhibit patterns that do not follow the branching events associated with this stage in embryogenesis and may reflect an early requirement for Hh in tracheal induction.

The number of tissue specific phenotypes associated with mutations in *hh* (compiled in Flybase, 2009) illustrates the diverse role Hh signaling plays in

development. It is not fully understood whether Hh signaling activates a small set of targets for redundant use in different tissues or whether gene activation is specific to the tissue in question. Probing whole genomes with cDNA microarrays has proven to be a useful tool in identifying differentially expressed genes for specific tissues or whole organisms. For the purpose of identifying Hh targets, arrays have been used to probe cell lines with stable Gli1 expression (Yoon et al., 2002). Arrays have also been used to ask how Hh controls a known process such as the proliferation of a neuronal sub-type in the brain (Oliver et al., 2003). The results from the DAMID experiments argue that Hh signaling activates diverse sets of targets depending on tissue type and that the number of genomic regions recognized by Ci numbers in the thousands. In the next section, I utilized cDNA microarrays to profile whole embryos that harbor loss of function mutations in the Hh signaling pathway. The expected result was that gene expression would change as a result of altering Hh signaling. By analyzing the transcriptional output of different mutated Hh signaling components with respect to wild type I hoped to separate noise that is inherent with array analysis and identify dedicated Hh targets that carry out development over a wide range of tissues. The expectation was that direct targets of Hh signaling would be identified by both DAMID and expression array analysis.

Hedgehog responsive genes

Although DAMID methylation identifies regions in the genome where Ci binds, binding is not necessarily synonymous with Hh-responsive genes. To complement the

DAMID experiments and to identify genes that are regulated by Hh signaling in stage 10-11 embryos, I assayed transcriptional levels by hybridization to genomic microarrays. Transcripts isolated from embryos with normal levels of Hh signaling were compared to transcripts isolated from embryos with either deficient or elevated levels. As a general strategy for analyzing the transcriptional profiles of embryos harboring null mutations for components of the Hh signaling pathway, I compared amplified mRNA from stage 10-11 homozygous *hh* mutant embryos to mRNA from their heterozygous siblings. Probes representing the heterozygous and homozygous mutant backgrounds were individually labeled with either Cy3 or Cy5, combined, and hybridized to arrays containing a spot for each of the ~13,600 predicted genes of the *Drosophila* genome. To help eliminate false positives, four different homozygous mutant backgrounds (*hh*, *ptc*, *smo*, and *ci*) were tested. In addition, I ubiquitously expressed the constitutively active Cim1-m4 mutant using the Gal4/UAS system (Brand and Perrimon, 1993) and compared the transcript levels to those of DaGal4 embryos.

To analyze the results of this mircoarray study, I applied a clustering algorithm to the microarray data to correlate Hh-responsive genes across the different genetic backgrounds. I made 3 systematic comparisons of the array data based on known observations of the Hh signaling pathway. First, since the primary role of Hh is to relieve inhibition by Ptc on the downstream signaling component, Smoothened (Smo), I asked if genes down-regulated in a Hh null background are also down-regulated in a *smo* null background. Second, loss of function *ptc* conditions should correlate with conditions in which Ci has been converted to a transcriptional activator. Thus, I compared the

transcriptional profile of *ptc* null embryos to embryos in which a constitutive Ci activator is expressed ubiquitously. Third, in order to reduce false positives, I compared the transcriptional profile of wt to *hh*, *ptc*, *smo*, and *ci* mutant backgrounds to determine which genes are up-regulated when Hh signaling is present or enhanced, or reduced when Hh signaling is absent. This approach assumes that all four genes function in a linear pathway to affect target gene transcription. From the three comparisons, I identified a list of non-redundant genes that respond in a positive way to Hh signaling.

hh vs. smo

Criteria that were used to identify Hh-responsive genes by the clustering analysis required transcription levels to meet or exceed a fold induction threshold in 4 out of the 9 experiments (Figure 6 columns). In this way, responsive genes that are specific to *hh* or *smo* will be included as sub-clusters, along with genes that satisfy the threshold for both backgrounds. Genes that were up-regulated in a *hh*+ or *smo*+ background are represented as shades of green while genes that were up-regulated in *smo* or *hh* loss of function backgrounds are represented as shades of red. Since the primary role of Hh is to relieve inhibition by Ptc on the downstream signaling component, Smo, I asked if genes commonly down regulated in a *hh* loss of function background are also down regulated in a *smo* mutant background with respect to wt. Figure 6 shows that this pattern holds true for the genes identified in stage 10-11 embryos. Under my screening conditions 79 genes organize into a sub-cluster (Figure 6 middle sub-cluster) that show a general transcriptional up-regulation in wt embryos compared to *hh*- and *smo*- mutant

backgrounds. As a confirmation of these findings, I asked if this method identified known targets of *hh* signaling that are activated during this window of embryonic development. Confirming the method as a valid technique for identifying Hh signaling targets, I observed up-regulation of *wingless* (*wg*), *roadkill* (*rdx*), *bagpipe* (*bap*), *drumstick* (*drm*), *Drop* (*Dr*), *mirror* (*mirr*) and *engrailed* in *smo*+ and *hh*+ backgrounds (asterisks). Notably absent from my analysis is the Hh target gene *ptc*. I attribute this as a failure to amplify *ptc* RNA and not a hybridization artifact, as several control spots for *ptc* also did not respond. Interestingly, 42 genes showed a general up-regulation in wt with respect to *smo*- embryos but not with respect to *hh*- embryos (Figure 6 top sub-cluster). Perhaps this is an indication of basal levels of Smo activity, capable of activating gene expression independently of Hh. Genes of interest in this category are *dally-like* (dlp) and *suppressor of hairy wing* (*su(Hw)*). Finally, 36 genes generally up-regulate in both *smo* and *hh* null backgrounds (Figure 6 bottom sub-cluster) suggesting they are normally targets of repression by Hh signaling. To date, the only evidence for Hh signal-induced transcriptional repression is the up-regulation of *ser* in the ectoderm (Alexandre et al., 1999) and the *ladybird* genes in cardiac mesoderm (Jagla et al., 1997); both are examples of genes that are de-repressed in hh mutants. *twist* (Figure 6) may fall into this category. *twist* is expressed in a subset of the mesoderm at this stage in development, while *smo* is strongly expressed throughout the mesoderm.

ptc vs. CiAct

Previous studies identified *ptc* as a negative regulator of the *hh* signaling pathway (Chen and Struhl, 1996). In the embryo, loss of *ptc* leads to the expansion of the *hh* signaling target *wg* (Ingham and Hidalgo, 1993), presumably due to the combined action of unrestricted movement of Hh protein through responsive tissue and constitutive formation of Ci activator. I performed the array assay on *ptc* null embryos to identify genes that are up regulated due to unrestricted Hh signaling. As a complementary experiment, I ubiquitously expressed a constitutively active form (Chen 1999) of Ci and checked the effect of both mutant backgrounds using arrays. Genes meeting or exceeding a fold induction threshold in at least 5 of the 9 arrays were identified by the clustering analysis. A sub-cluster of 75 genes satisfied these criteria for both *ptc*-/- and DaGAL4>UAS CiAct (Figure 7). Among these 75 genes are the known Hh targets *wg*, *drm*, *rdx*,and Cyclin E, confirming the analysis as a valid measure for identifying Hh targets. The identification of *ci* as a putative target was unexpected, as no previous study has suggested that it is. As described below, I analyzed the responsiveness of *ci* to Hh signaling in depth and confirmed that Hh does regulate *ci* expression. Finally, a gene that is up regulated in *ptc*- and Da>CiAct embryos is Casein kinase1 (CK1), a Ser/Thr kinase that phoshorylates full length Ci for efficient processing to repressor.

hh, smo, ptc and ci array overlap

The generally accepted model for Hh signaling posits that the components of Hh signaling function as a linear pathway to ultimately affect the status of Ci as a transcriptional regulator. If this is the case, then the genes affected by mutating different components of the pathway should be similar overall. I have shown previously that there

are genes commonly regulated by *hh* and *smo* and similarly shown that there are genes commonly regulated by *ptc* and *ci*. I tested the ability of four pathway components to regulate the same genes by performing a clustering analysis on array data from independently generated embryo samples of *hh*, *ptc*, *smo*, and *ci* null backgrounds. Two arrays from each mutant background were included in the analysis. For this comparison, genes identified by the clustering analysis were required to show a threshold fold up-regulation in 4 out of the 8 arrays. In this way, genes responding to a subset of backgrounds with respect to wt will be allowed to segregate into sub-clusters if they meet or exceeded the threshold in at least two of the mutant backgrounds tested. The results are shown in Figure 8. Genes down regulated in *hh*, *smo*, and *ci* null embryos with respect to wt are shown in green while genes up-regulated in *ptc* null embryos with respect to wt are shown in red. 99 genes group into a sub-cluster with a positive response to *hh* signaling across all backgrounds (bottom sub-cluster). Two genes of this sub-cluster with duplicate spots noticeably show a down regulation in wt with respect to *hh*- embryos. A smaller sub-cluster represents 3 genes that also show a positive, consistent response to Hh signaling compared to all genetic backgrounds using the expression array assay: *gsb*, *hh*, and *wg* (asterisk). Interestingly, two other sub-clusters show a response that is specific to *hh* and *smo* (27 genes, sub-cluster second from top) while another shows a response that is primarily specific to *ci* and *ptc* (23 genes, 3rd subcluster from top). The unexpected result that Hh target gene expression could be affected by loss of one pathway member but not another is difficult to reconcile unless a significant level of basal activity exists for specific pathway members (see discussion).

Intersection of Dam ID and expression array targets

From the three expression array comparisons I identified a total of 199 non-redundant "target" genes that are up regulated when Hh signaling is present or enhanced in the embryo. I cross-referenced these Hh responding genes against the DamCiAct results to determine if there were Ci binding regions nearby or in the responding transcription units. Based on the occurrence of DAMCiAct binding regions in/nearby the ~13472 genes of the Drosophila genome, I expect 26% of the target genes to have a DAMCiAct binding region based on chance alone. Among the 199 target genes, I observe DAMCiAct binding regions at a rate of 45% (50% increase over chance). The high rate of overlap between genes associated with Ci binding regions and transcriptional up-regulation due to Hh signaling provides a list of high confidence target genes.

Tissue-specific response to CiAct

The set of genes identified by the DAMID and expression array analyses suggests that the Hh signaling pathway functions in a diverse range of tissue types. Moreover, wildtype expression patterns of many of the genes in question confirm they are activated in tissue-specific populations of cells rather than being induced throughout the embryo. I tested the response of putative Hh targets to CiAct to determine the extent to which these genes are respond to Hh signaling. By comparing the response to ubiquitously expressed CiAct with the normal domains of *hh*, *ci*, and Ci activation, I defined the limits of Hh signaling competence in different tissues.

Dorsal ectoderm

I expressed CiAct under control of the ubiquitous *daughterless* Gal4 driver and assayed the transcriptional response of putative target genes by *in situ* hybridization to whole mounts. The *ptc* gene is thought to be a universal *hh* target, in the sense that its expression is activated in all cells that receive the Hh signal. The predominant pattern of *ptc* expression in stage 11 embryos is two ectodermal stripes, both immediately adjacent to the *en*/*hh* expression domain, one anterior and one posterior. Thus, *ptc* expression marks cells that are immediately anterior to the para-segmental border as well as cells that comprise the most anterior part of the adjacent segment. The dorsal-most portion of the anterior *ptc* stripe in each segment has a higher level of expression than its sister stripe (Hidalgo and Ingham, 1990; Figure 9A arrow.) Upon ubiquitous expression of CiAct, I observed a dramatic *ptc* response in virtually all cells of each epidermal segment, including up-regulation at the dorsal part of the anterior stripe (Figure 9E). I next tested whether other *hh* targets that are normally expressed in the dorsal ectoderm are capable of such a response.

Three members of the *odd-skipped* family of genes, *drm*, *sob* and *odd*, are expressed in nearly identical, segmentally-repeated patterns at stage 11 (Figure 9 B-D blue staining). *drm*, *sob* and *odd* genes are located immediately adjacent to each other on chromosome arm 2L, while the fourth family member, *bowl*, is located approximately 165kb downstream; *bowl* has a different expression pattern (Fig 9 Top and BDGP expression patterns). Significant DAMID signal at *drm*, *sob* and *odd* is localized and

robust, with three regions of overlapping DAMICiAct and DAMCiRep signal. These areas tend to be centered near clusters of Ci binding motifs (Figure 9 Top, red squares). Each dorsal ectoderm stripe of *drm*, *sob* and *odd* was immediately posterior to the *hh* LacZ stripe, which marks the Hh signaling domain in these double labeled embryos (Figure 9B and not shown). A spot of expression was also present more ventrally, but this domain was not connected to the dorsal stripe (out of focal plane). *drm*, *sob* and *odd* each responded to ubiquitous CiAct by increasing their expression domain from 2-3 cells to 5-6 cells in the posterior direction relative to the *hh* Lac Z (Figure 9 F). In contrast to *ptc*, the expansion of expression of the *odd-skipped* family genes in *Da*>CiAct embryos extended only partially into the domain of *ci* expression (See figure 10A) and did not reach full activation throughout the segment. This suggests that Hh signaling is not sufficient to activate *drm*, *sob* and *odd* in all cells of the segment. Moreover, the result that *drm*, *sob* and *odd* are not up-regulated by CiAct in other tissue systems where Hh signaling is also active (not shown), suggests that any putative combinatorial regulation by Ci at the *drm*, *sob* and *odd* enhancer(s) requires limiting tissue-specific factors.

Tracheal placode

Glazer and Shilo (2001) reported a direct role for Hh signaling in the branching of the primary nodes of the early tracheal system. They showed that *ptc*-LacZ is activated in cells that abut the anterior portion of the tracheal placodes and that Hh signaling is necessary and sufficient for enhancer trap expression of a gene expressed in those cells. In addition, *hh* mutant embryos fail to initiate the early migration programs that define the branches of the tracheal system. Here, we extend those analyses by analyzing *ptc*

expression in the context of Ci protein stability (Ci protein is stabilized in cells that transduce the Hh signal.), and the expression of a novel Hh target gene in the tracheal placodes, *ImpL2*.

In agreement with Glazer and Shilo (2001), we found that *ptc* expression was limited to a stripe of cells corresponding to the anterior portion of the invaginating cells of the tracheal placodes in stage 11 embryos (Figure 10C). At this time, *ci* RNA is expressed in cells on both sides of the tracheal placode as well as the rest of the segment leading up to the *engrailed* domain where it is deactivated (Eaton and Kornberg, 1990). Given the expression of the Hh target *ptc*, it is surprising that stabilization of full-length Ci protein, a hallmark of Hh signaling activity, occurs in cells on both sides of the placode and not just the anterior side (Figure 10B). This suggests that *ptc* expression is activated in a subset of cells that are capable of responding to Hh. DAMID analysis (Figure 10J) and expression array analysis (Figure 6) led to the idenfication ImpL2, a novel Hh target gene expressed in the developing trachea. Like *ptc*, *ImpL2* expression in the tracheal placode is highest in anterior cells (Figure 10E) and continues ventrally in a stripe (not shown). Unlike *ptc*, *ImpL2* is expressed in the dorsal-most cells of the placode at high levels(Figure 10E). Given its highly restricted expression pattern in the tracheal system, we assayed *ImpL2* expression in embryos expressing ubiquitous CiAct to determine its full range of Ci mediated activation. *Da*Gal4 CiAct embryos showed a marked increase in the range of *ImpL2* expression in the ectodermal cells immediately dorsal to the placode (Figure 10F bracket). These cells are within the normal domain of *ci* RNA expression (compare Figure 10F with 10A). In addition, *ImpL2* was up-regulated

in the ventral ectoderm in these embryos (not shown), implying the presence of two separate regulatory elements that are sensitive to Ci.

Similar ectopic expression in the dorsal ectoderm was observed when CiAct was driven in all tracheal cells by *btl*Gal4 (Figure 10G). In these embryos, expressed was restricted to the dorsal trunk and transverse connective in germ band retracted embryos (10G inset).

These results suggest that Hh signaling is normally capable of activating *ImpL2* in only a subset of tracheal cells that transduce the Hh signal. In addition, ubiquitous or pan-tracheal CiAct induces *ImpL2* in cells seemingly competent to respond, but this competence does not extend to all tracheal cells. This evidence argues for a tracheal specific factor that cooperates with Ci to induce *ImpL2* expression outside of its normal domain. Embryos lacking *hh* exhibit reduced but not a complete loss of *ImpL2* at stage 11 compared to wildtype (Figure 10H). During later stages, retracting *hh* mutant embryos continue to express *ImpL2* in the developing trachea (not shown). This phenotype could be attributed to an early but not late requirement for Hh signaling or could reflect multiple positive regulatory inputs for *ImpL2* expression. Two binding regions for Ci at the *ImpL2* locus were identified in the DAMCiAct study, one in the first large intron of the gene and one immediately downstream of the transcription unit (Figure 10J). Both binding regions contain consensus Ci biding sites (red lines). Consistent with *ImpL2* tracheal expression, we found binding sites for *vvl* and *trh* near the consensus Ci binding

motifs. Both Vvl and Trh are transcription factors that previous studies have implicated in tracheal development (Murphy et al., 1995; Wilk et al., 1996; Boube et al., 2000)

Embryonic visual primordia

Hh signaling plays a role in the development of the embryonic visual system which is comprised of Bolwig's organ (larval eye) precursor cells and the embryonic optic lobe. *hh* is expressed in and around Bolwig's organ precursor cells in the posterior optic lobe; *ptc* expression is localized to the anterior optic lobe (Chang et al., 2001). Increased Hh signaling provided by heat shock or by loss of Ptc activity results in a cyclyopic optic lobe. This phenotype is characterized by optic lobe tissues in the dorsal ectoderm, fusing the normally bilateral visual system (Chang, 2001).

In addition to *hh* and *ptc*, I detected the expression of three other Hh signaling components in the optic lobe primordia: *ci*, *smo*, and *rdx* (Figure 11 B,C, and F). Although *ci* RNA is present in both the anterior and posterior lips of the optic lobe (11B) I found that stabilization of full-length Ci protein as well as refinement of *rdx* expression, a target and negative feedback regulator of Ci, was mainly in the posterior lip (Figure 11 E and F arrows). Consistent with this finding, I identified four additional putative Ci target genes expressed in the developing optic lobe, two of which refine exclusively to the posterior by embryonic stage 12 (Figure 12 *drl* and *eya* A,B). The other two are expressed in both the anterior and posterior lobes (Figure 12 *aop* and *sna* C,D). I analyzed the response of these genes to ubiquitous CiAct in the developing visual system. In all cases, the response was localized activation of expression extending from the

posterior optic lobe into the dorsal ectoderm of the head, reminiscent of the Hh-induced cyclopia phenotype (Chang, 2001 Figure 12 A''-D'). In these embryos, expression was unchanged in the optic lobe primordia (Figure 12 A'-D' arrows). Because the response to CiAct occured in a stripe of cells in the visual system, I conclude that Ci requires the combined activity of a tissue specific factor to activate gene expression in this region.

Ci is a hedgehog signaling target

The three vertebrate homologs of *ci*, Gli1, Gli2, and Gli3, each have different activities with respect to transcriptional activator and repressor function (Aza-Blanc et al., 2000). Gli1 has been shown to be primarily an activator of transcription and is itself a transcriptional target of Sonic hedgehog (Lee et al., 1997). Here, I show that *ci* is a target of *hh* signaling. First, using expression arrays, I observed up-regulation of *ci* transcript levels by at least 1.7 fold in embryos mutant for *ptc* (see Figure 7). This result is a likely reflection of an overall increase in Hh target levels due to unrestricted signaling (Ingham and Hidalgo, 1993). Second, I observed a distinct DAMCiAct peak overlapping the transcriptional start site of *ci* (Figure 13A). Third, until germ band retraction commences, wild type expression of *ci* was homogeneous throughout each segment (Eaton and Kornberg, 1990; Motzny and Holmgren, 1995). However, embryos older than stage 11 had graded expression of *ci* RNA in the ectoderm; the highest levels of *ci* were present in cells that receive the Hh signal (Figure 13B arrow). Heterogeneous levels of *ci* were disrupted by driving Hh ubiquitously, resulting in high levels of *ci* throughout the segment (Figure 13D). The increased level of *ci* expression appeared as an additional stripe in the posterior part of the segment because of a cleft that formed in the middle of

each segment. The mechanism for the Hh induced cleft formation is unknown; it may relate to the role of Hh in segment groove formation (Larsen et al., 2003). Finally, *ci* expression in the wing disc did not appear uniform as previously reported (Slusarski et al., 1995). Ci expression is limited to the anterior compartment due to repression by *en* in the posterior (Eaton and Kornberg, 1990). In wildtype third instar wing discs, *ci* expression was graded, with highest levels at the A/P compartment border (Figure 13C bracket) in cells closest to the source of Hh. Taking these results together, I conclude that *ci* is a target of Hh signaling and this may reflect a conserved mechanism that contributes to the regulation of Hh signal transduction.

Table 1*: DamID statistics*

	DamCiRep	DamCiAct
p-value cutoff	.02	.001
# Binding regions (BR)	1743	2438
Median BR length	993	2133
Nearby genes	2747	3521
	66% of nearby genes intersect with DamCiAct	52% of nearby genes intersect with DamCiRep
Fold enrichment of TGGGTGGTC within BRs	2.42 (p-value=7.2e-09)	2.55 (p-value= 4.0e-24)

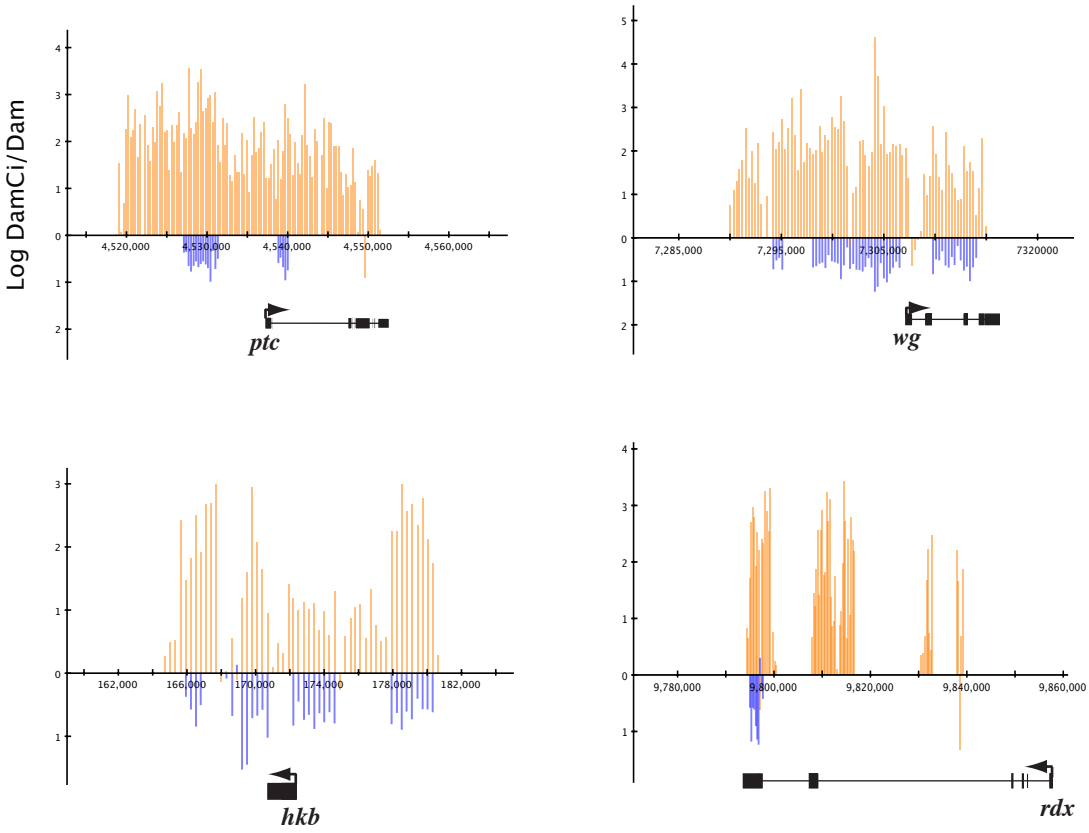

Figure 1: DAMID proof of principle. Loci of 4 known Hh targets genes are shown with DAMID intensityvalues on the y axis with respect to genomic coordinates on the x axis. DAMCiAct signals are represented asorange bars, DAMCiRep signals are represented as blue bars.

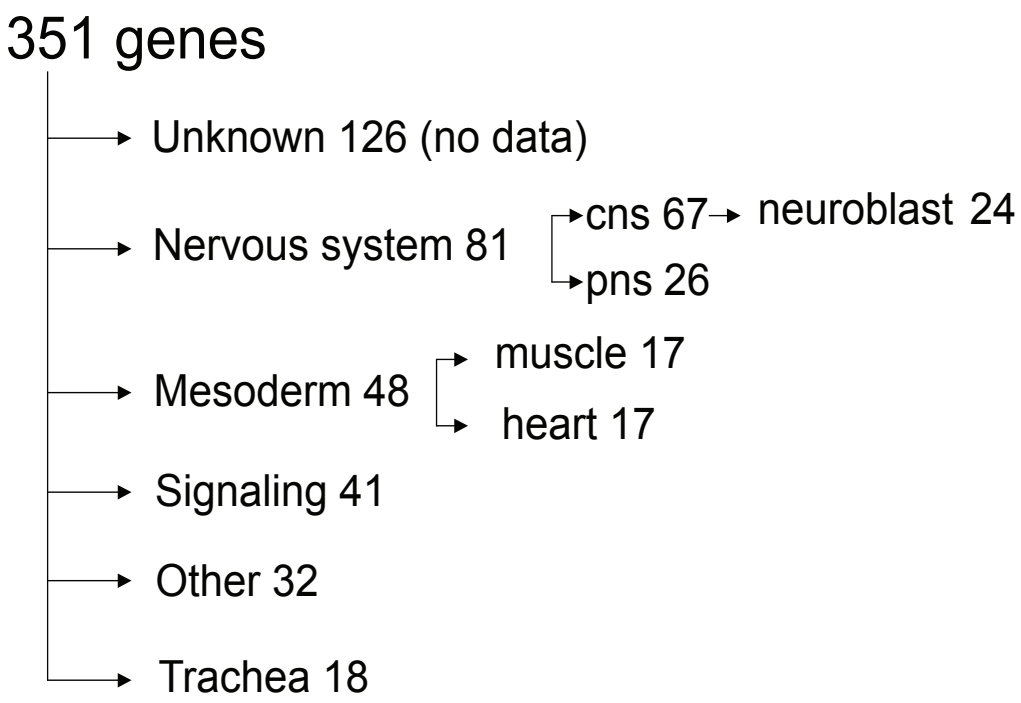

Figure 2: Functional categorization of genes associted with significant overlapping DAMID signal.

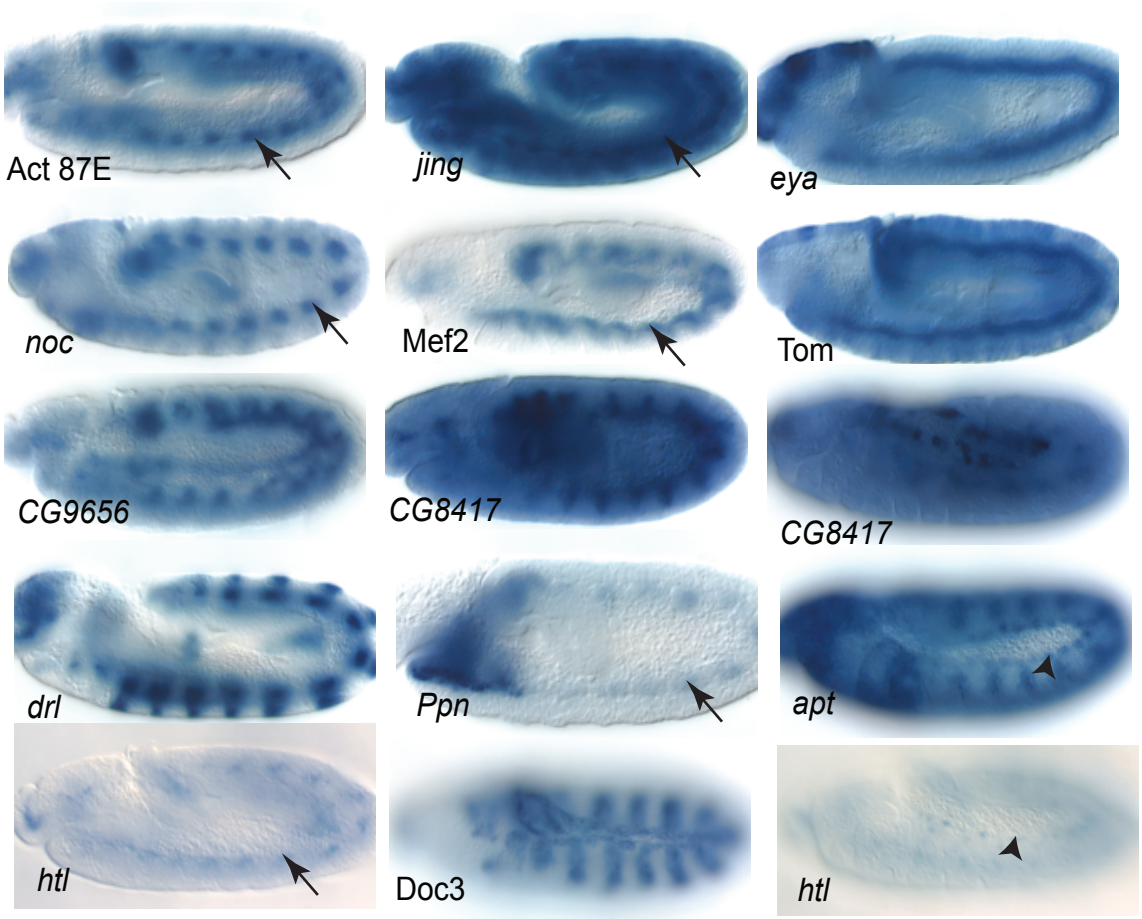

Figure 3: WT expression patterns of mesoderm genes associted with DAMID.
Several genes are expressed in the visceral mesoderm (arrows) where Hh signaling isknown to play a role in patterning. Other genes are expressed in mesodermalderivatives such as heart precursor cells (arrowheads).

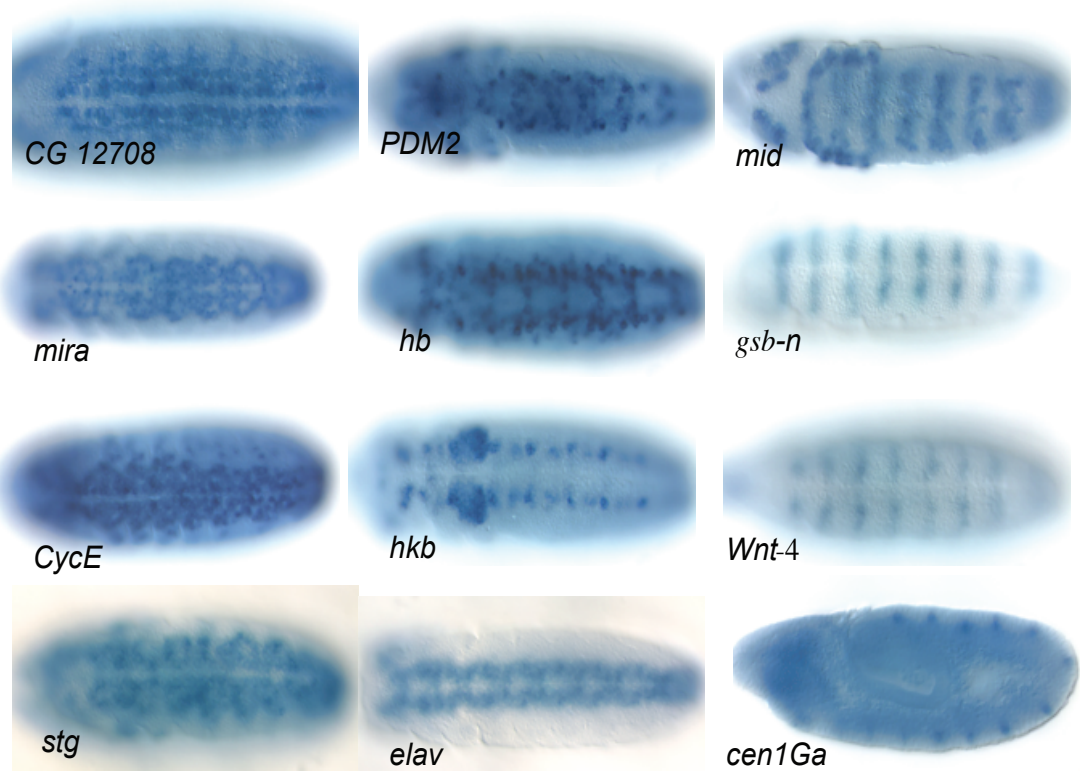

Figure 4: Expression patterns of nervous system genes associated with DAMID.
Shown are expression patterns falling into two classes: 1) expression in sub-setsof neuroblasts (first two columns) or 2) patterns of striped expression (last column).

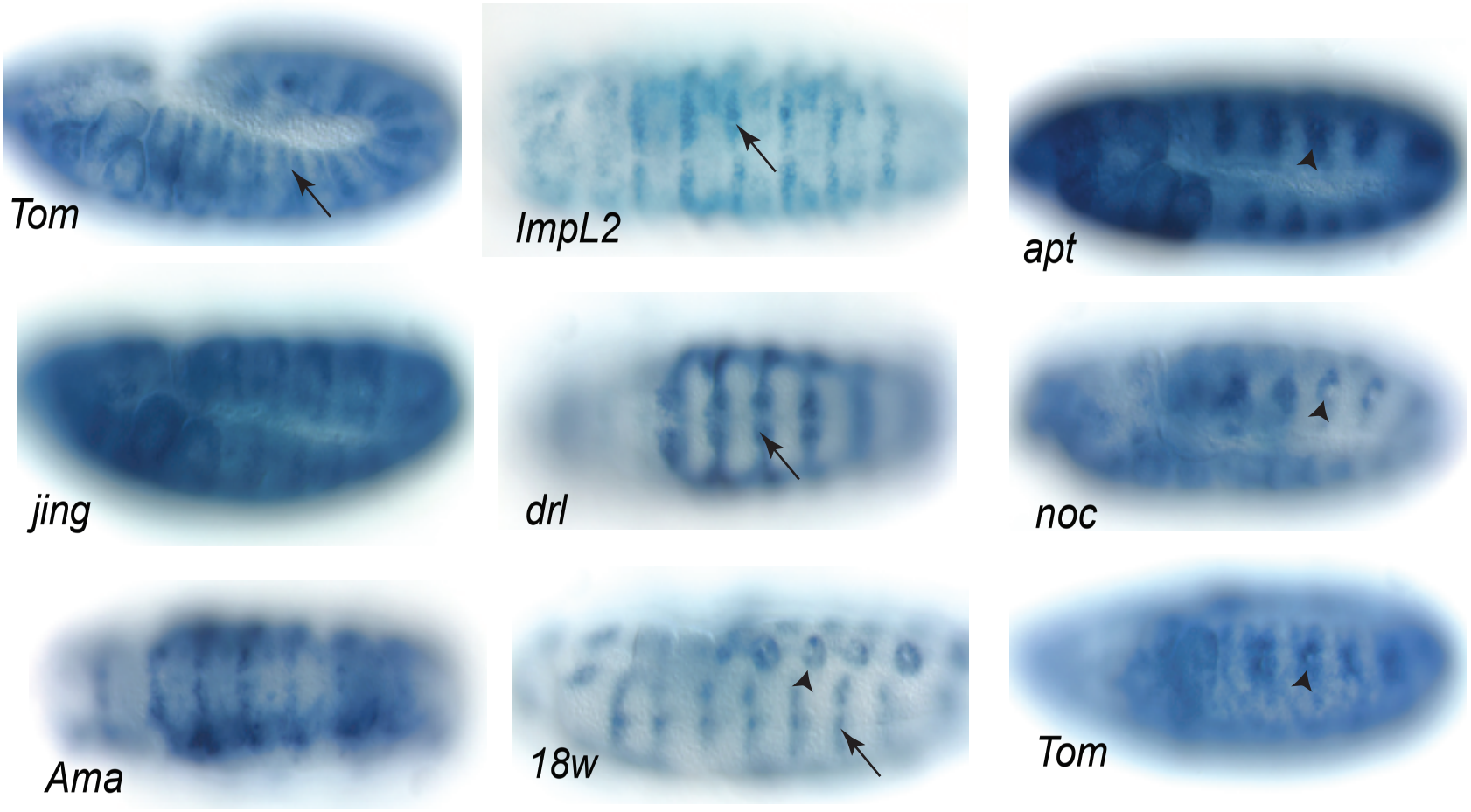

Figure 5: Ectoderm genes associated with DAMID. Striped patterns of expression found in the Hh signaling domain (arrows) or expression found in the tracheal placodes (arrowheads).

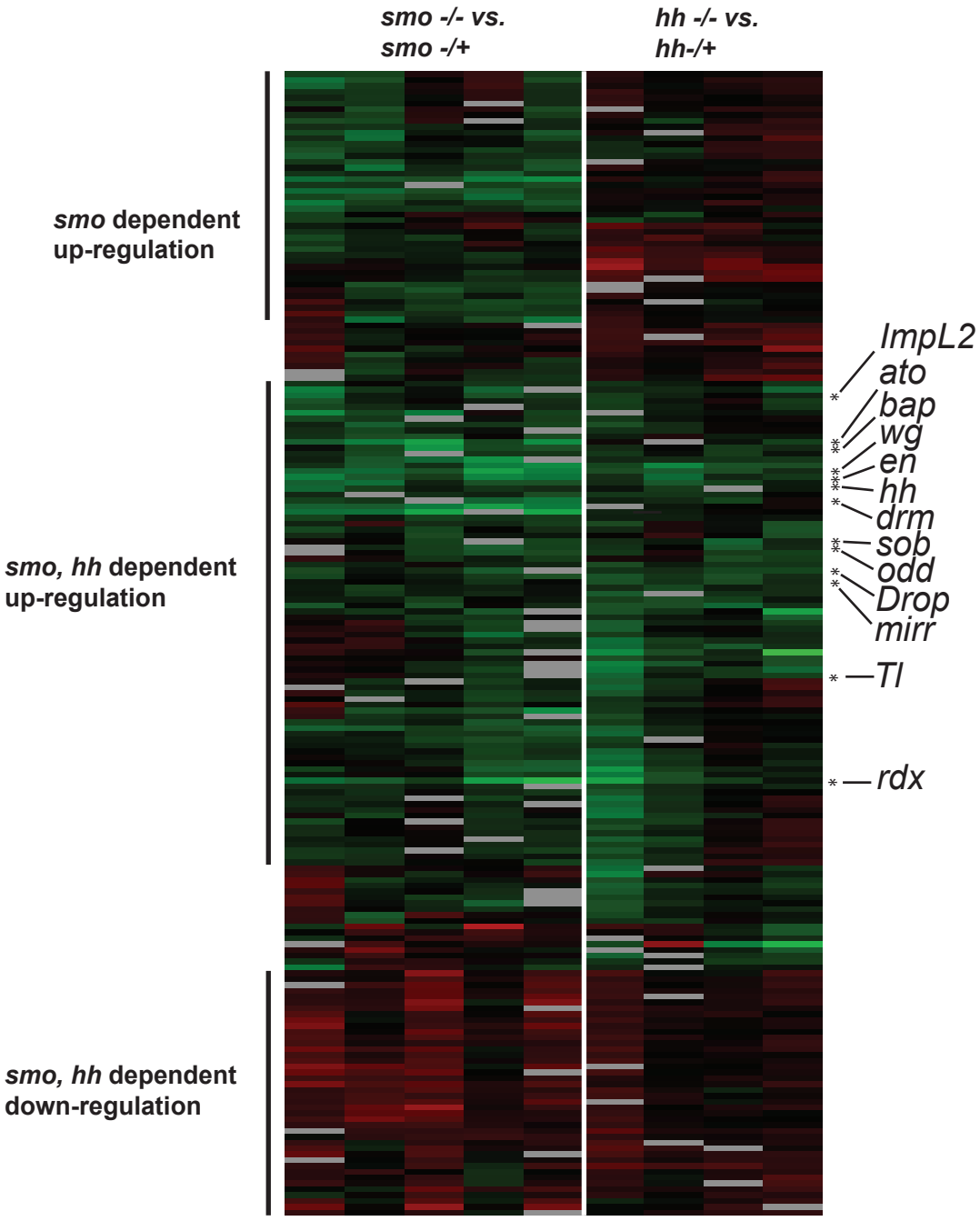

Figure 6: Cluster analysis of *smo* and *hh* null embryos.
Intensity values for genes (rows) up-regulated in wt embryos compared to *hh-* and *smo-* embryos (columns) are represented as dark green for low values and bright green for high values. Known and novel targets of Hh signaling are listed by gene name.

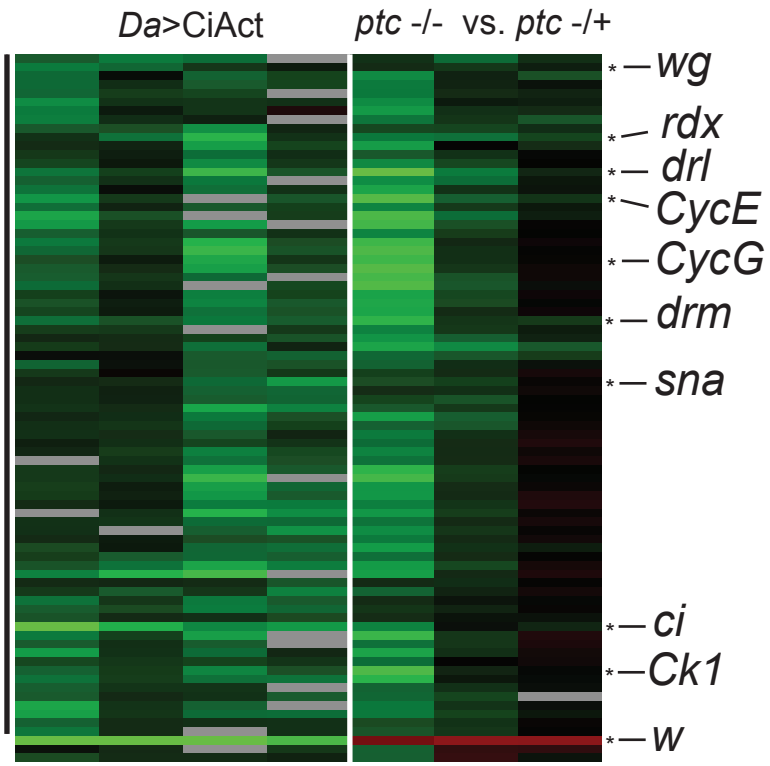

Figure 7: Clustering analysis of genes responding to *Da*>CiAct and *ptc*- backgrounds.

A sub-cluster of genes up-regulate in response to both genetic backgrounds revealing several known Hh signaling targets as well as genes characterizedby this study. In addition,*Ck1* is identified, a negative regulator of Hh signaling.

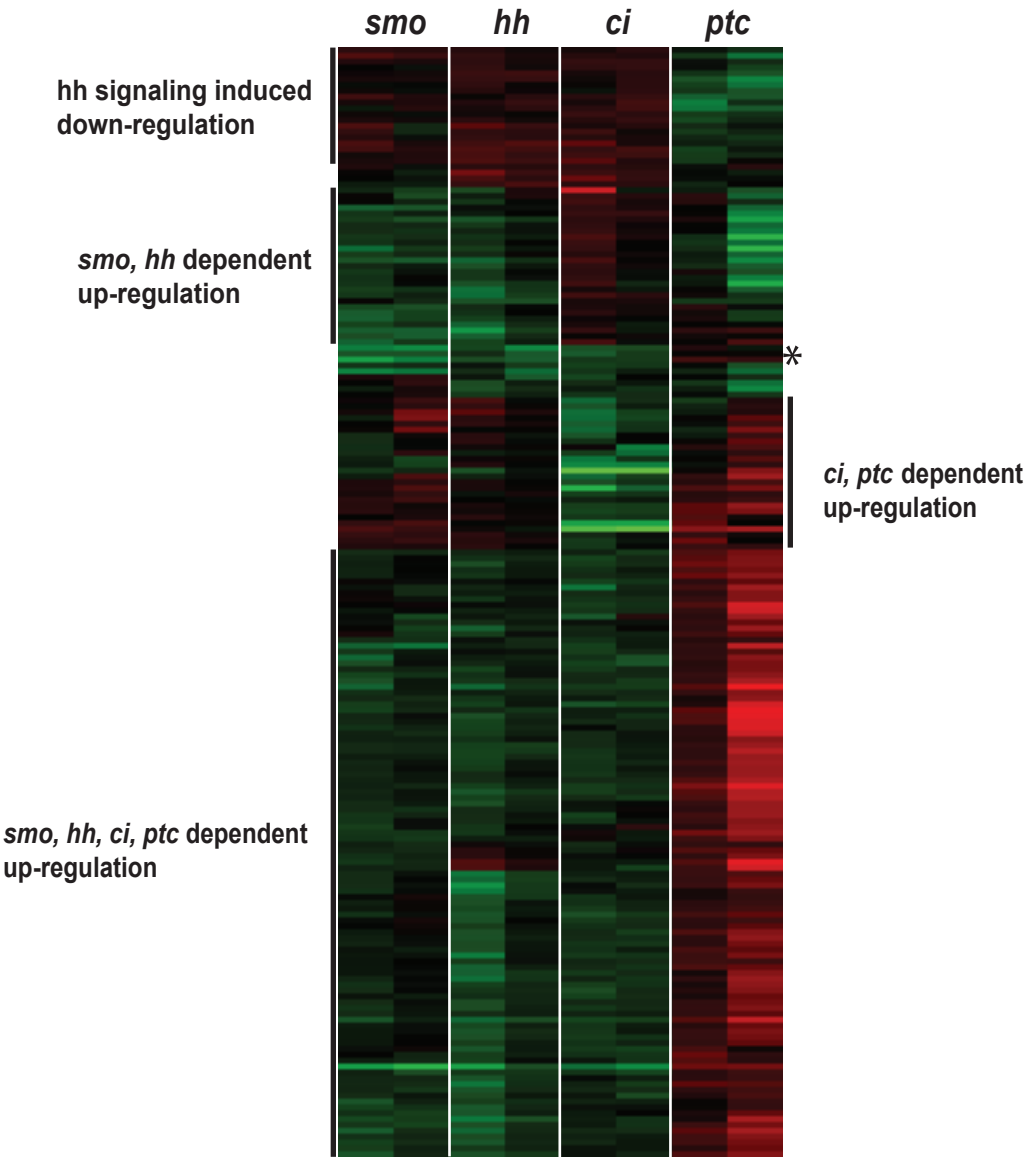

Figure 8: Clustering analysis of 4 Hh pathway components.
Comparison of *smo*, *hh*, *ci*, and *ptc* loss of function embryos to wt reveals patterns of gene activation specific to genotype.

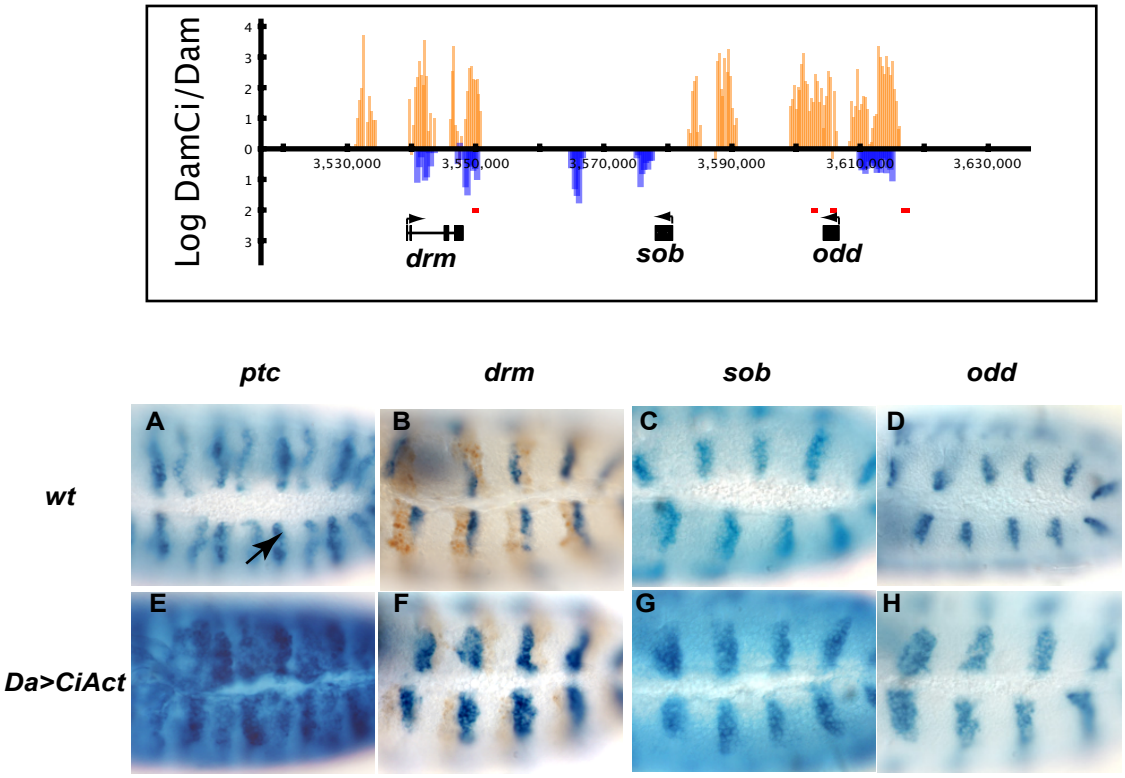

Figure 9: Dorsal ectoderm genes are up-regulated in response to ubiquitous CiAct.

Top: the arrangement of *drm*, *sob*, and *odd* transcrption units on chromosome 2L. Significant DAMID signal is present at discrete locations in the region (DAMCiAct orange bars, DAMCiRep blue bars) roughly associatingwith Ci binding motifs (red boxes). Bottom: Embryos are arranged anterior left and dorsal up. wt expression of *drm*, *sob*, and *odd* is localized to the Hh signaling domain in the dorsal ectoderm (B-D) concordant with elevatedptc levels (A arrow). Ubiquitous CiAct causes an expansion of the *drm*, *sob*, and *odd* stripes in the posterior direction (F-H) while ptc expression nearly fills the segment (E).

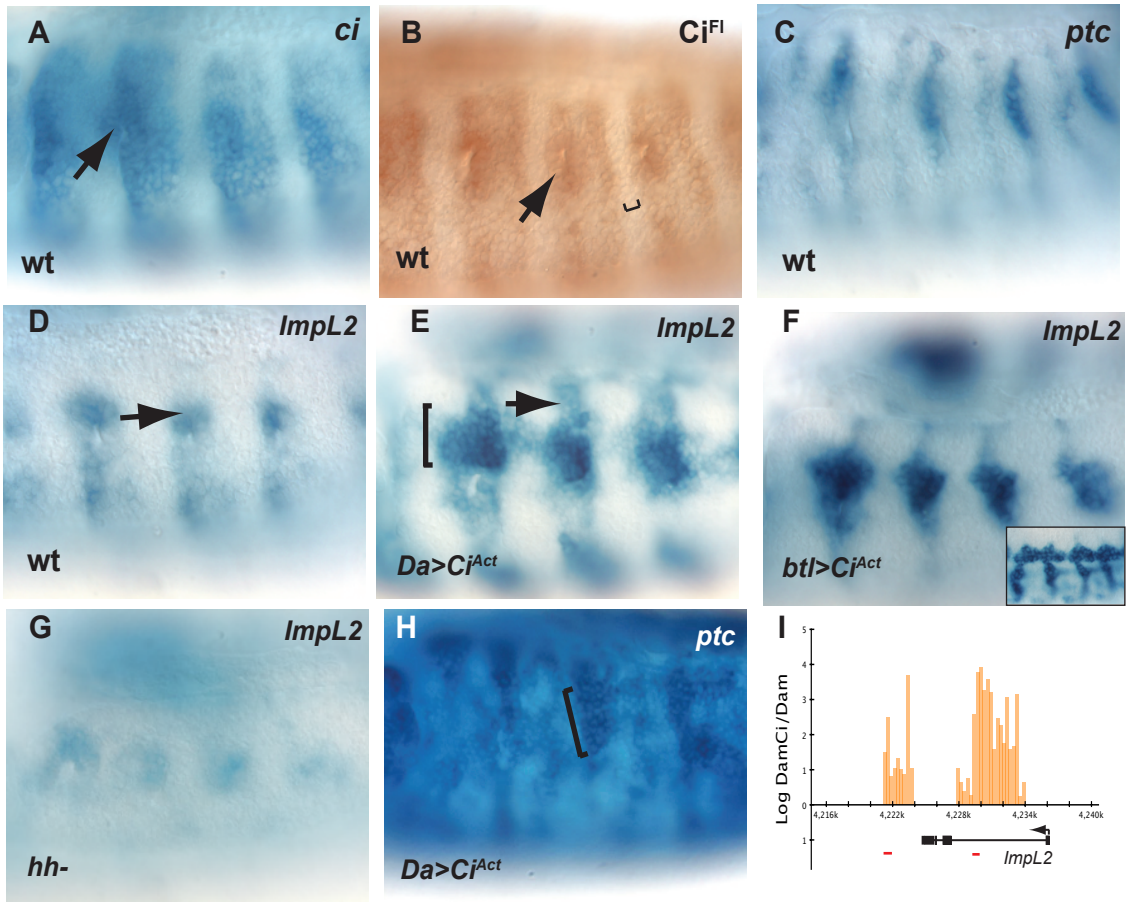

Figure 10: Tissue-specific regulation of lmpL2 in the tracheal placode.

Embryos are oriented anterior left.(A) *ci* expression is present throughout most of the segment and the tracheal placode (arrow). Stabilization of full length Ci protein occurs in stripes on either side of the *en* domain (B bracket) and in the tracheal placode (B arrow). *ptc* expression the early trachea is limited to the anterior portion of the placode (C). The *lmpL2* gene is associated with two regions of DAMCiAct binding and Ci bindingmotifs (J orange bars and red lines). *lmpL2* is expressed at high levels in theanterior and dorsal parts of the tracheal placode (D arrow).In *Da*>CiAct embryos *lmpL2* expression expands approximately 7 celldiameters into dorsal tissues (E bracket). A similar phenotype is observed if CiAct is driven throughout the tracheal system with *btl Gal4* (F) and refines to the dorsal trunk and transverse connective in germ band retracted embryos (inset). *lmpL2* expression is severely reduced in *hh-* embryos (G).(H) *ptc* expression in *Da*>CiAct embryos results in ectopic ptc in cells immediately dorsal to the tracheal placodes (H bracket).

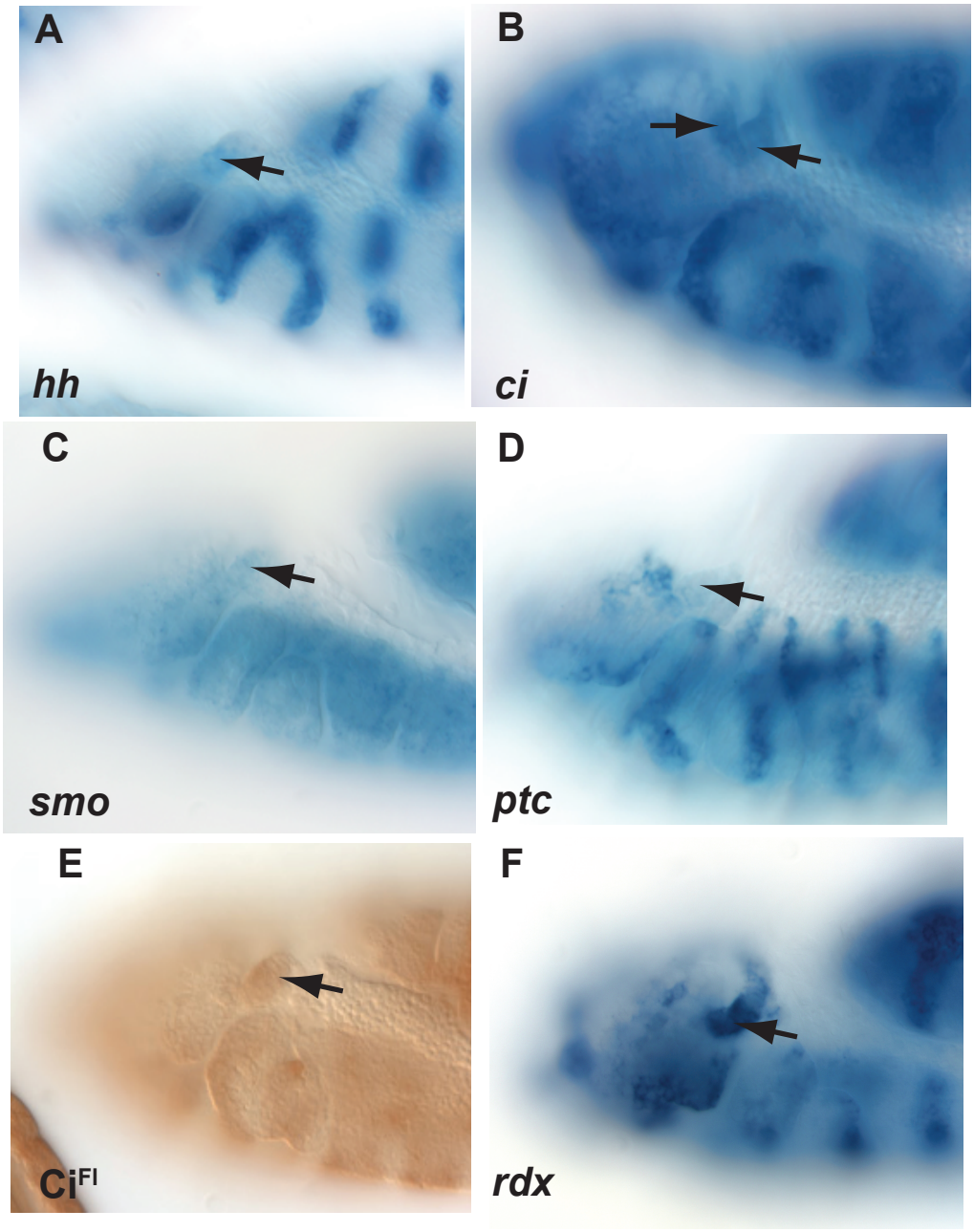

Figure 11: Expression of Hh signaling pathway components in the embryonic visual primordia. All embryos are viewed laterally, anterior to the left. *hh* and the Hh signaling target *rdx* are expressed in the posterior optic lobe (POL) (A and F arrows). *ci* expression is present in both lips of the optic lobe (B arrows) while *smo* is expressed predominantly in the POL (C arrow). The POL is largely devoid of ptcexprssion (D) even though Ci protein activation occurs mainly in the POL (E).

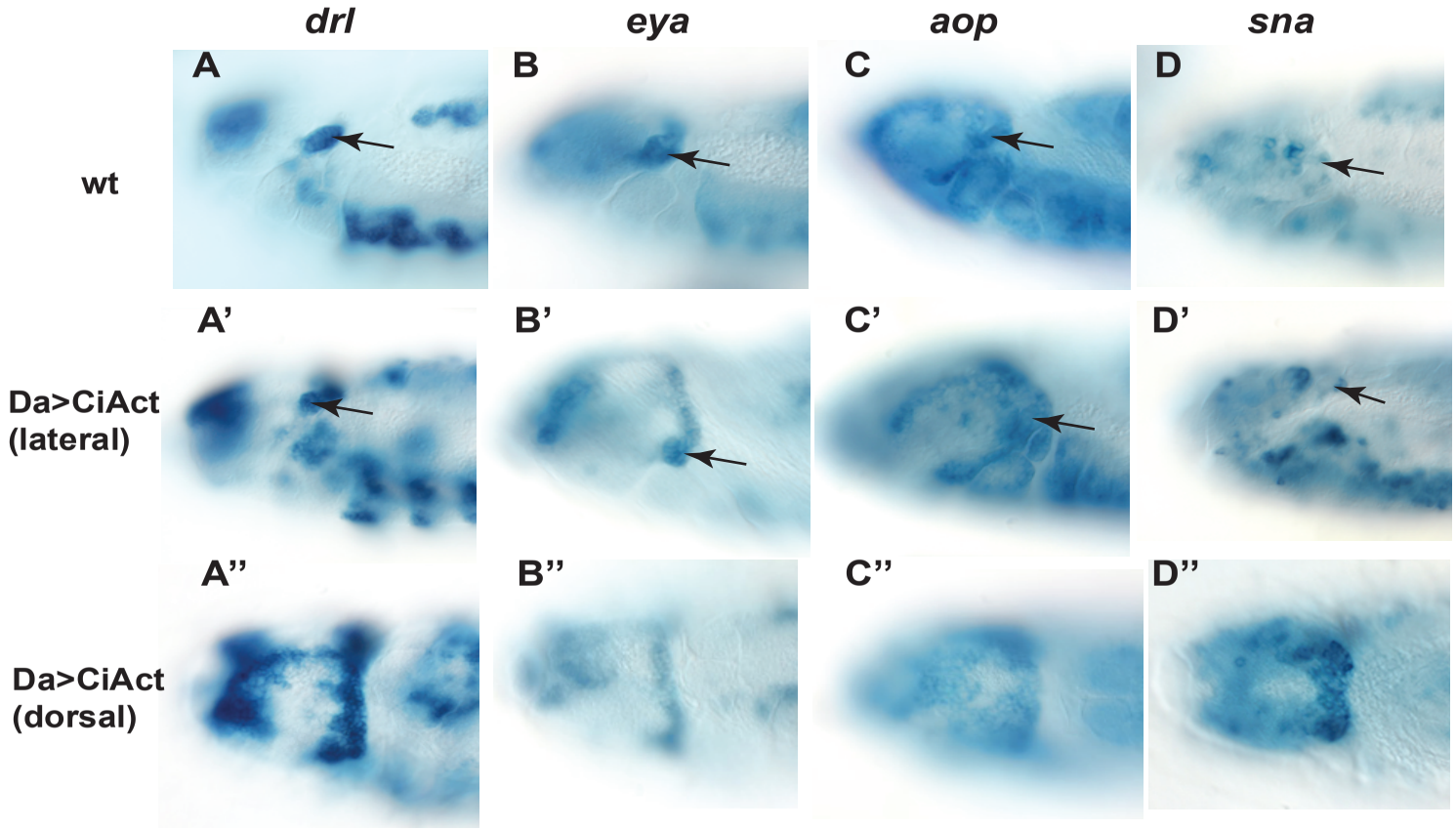

Figure 12: Response of visual primordia genes to ubiquitous CiAct.
Embryos are oriented anterior to the left, A-D and A'-D' lateral view. A''-D'' dorsal view. drl and eya expression is confined to the posterior optic lobe in wt embryos (A and B arrows) while *aop* and *sna* expression is present in the posterior (C and D arrows) as well as anterior optic lobe. In embryos expressing ubiquitous CiAct all markers are normally expressed in the lateral visual system (A'-D') while dorsal views (A''-D'') reveal ectopic expression of all markers in the dorsal ectoderm.

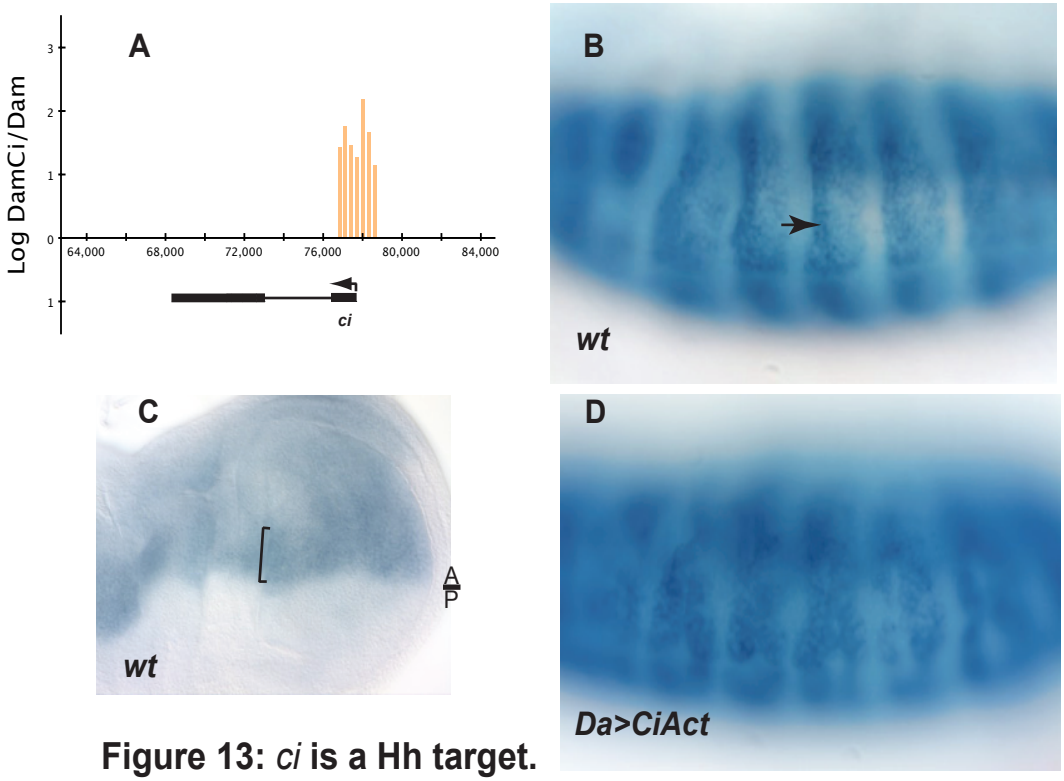

Figure 13: *ci* is a Hh target.
Embryos are oriented with anterior to the left. Signal from DAMCiAct experiment overlaps with the transcription start site of *ci* (A). wt expression of ci in stage 12 embryos shows an up-regulation in cells receiving the Hh signal (B, arrow). (C) *ci* expression in third instrar wing discs is enhanced near the A/P border (bracket). In (D), *ci* expression is up-regulatedin cells posterior to the Hh signaling domain.

Chapter 3

Discussion

The diverse effects of Hh signaling are mediated via signal-induced gene activation, however, many of the molecular details of the process are not well understood. Among the core principals that have been clearly established are: 1) Hh moves from cells that synthesize and activate it to signal to cells that do not make Hh (Tabata and Kornberg, 1994); 2) Hh signal transduction is effected through a receptor, Patched (Ptc) (Chen and Struhl, 1996; Ingham et al., 1991; Lu et al., 2006) and Smoothened (Smo), a seven-pass transmembrane protein whose localization, stability and activity are dependent upon the ligand bound state of Ptc (Alcedo et at., 1996; Denef et al., 2000; Zhao et al., 2007), and appears to regulate a complex of proteins that determine the localization and state of the transcription factor Cubitus interruptus Ci (Aza-Blanc et al., 1997; Jia et al, 2003; Ruel et al., 2003; Ruel, 2007). Ci activities mediate all transcriptional outputs of Hh signaling (Methot and Basler, 2001) and are manifested by a repressor form that is produced in the absence of Hh signaling and an activator form that is generated in response to Hh signaling (Aza-Blanc et al., 1997, Methot and Basler, 1999). It is the goal of my work to better understand the mechanism of Ci action.

Hh signaling is best understood in the context of the Drosophila wing imaginal disc. Hh is expressed specifically and only in the posterior compartment cells under the positive regulation of Engrailed (En) and it moves into the anterior compartment to endow cells across the Anterior/Posterior compartment border with properties of a

developmental organizer (Tabata and Kornberg, 1995). Ci expression is limited to the anterior compartment due to negative regulation by En (Eaton and Kornberg, 1990). Since Posterior compartment cells lack Ci, they are incapable of a transcription response to Hh. In contrast, all Anterior cells express Ci and cells that do not receive Hh convert Ci to its repressor form. In contrast, the Ci protein in cells at the A/P compartment border is not converted to repressor and instead can be activated by Hh signaling. This elegant genetic network effects the precise temporal and spatial regulation to Hh signaling.

In the wing disc, relative levels of Ci activator to Ci repressor are thought to be responsible for activating non-uniform domains of Hh target gene expression (Strigini and Cohen, 1997; Aza-Blanc et al., 1997; Methot and Basler, 1999). How the promoter regions of Ci target genes detect and respond to different levels of Ci activator vs. Ci repressor protein is unknown. Evidence suggests that Ci activator and Ci repressor can function through the same generic binding sites and that the response to either form of Ci is promoter specific (Muller and Basler, 2000). It is unknown, however, whether this is true for all Ci target genes.

The search for the components of Hh signaling as well its targets has been an intense area of study and has utilized biochemical, genetic, and genomic screens. The *Drosophila* system is amenable to direct screens for mutant phenotypes (Nusslein-Volhard and Wieschaus, 1980) as well as genetic modifier screens (Haines and van den Heuvel, 2000; Collins and Cohen, 2005; Casso et al., 2008). In addition, biochemical studies utilizing RNA interference screening for modulators of reporter activity have

been used (Lum et al., 2003; Nybakken et al., 2005). Both genetic and genomic screens have uncovered novel targets of Hh signaling. For example, a screen for modifiers of the Retinoblastoma pathway in Drosphila eyes led to the discovery of CyclinE and CyclinD as targets of Hh signaling (Duman-Scheel et al., 2002). The advent of cDNA microarray technology allows the probing of whole genomes for differentially expressed genes in specific tissues or whole organisms. For the purpose of identifying Hh targets, arrays have been used to probe cell lines with stable Gli1 expression (Yoon et al., 2002). Arrays have also been used to ask how Hh controls a known process such as the proliferation of a neuronal sub-type in the brain (Oliver et al., 2003). Despite these many efforts, only a small number of direct Hh targets have been identified to date.

The effects of Hh signaling on development are many and varied when one considers the phenotypes associated with mutations in the Hh gene (Compiled in Flybase, 2009). As these phenotypes are characterized, it is becoming increasingly clear that Hh functions to activate non-overlapping targets that are tissue specific. The obvious exception to this is the activation of *ptc* and *roadkill* (*rdx*) (Kent et al., 1996; Zhang et al., 2006) which appear to be up regulated in all Hh responding tissues to exert negative regulatory feedback loops. Examples of tissue specific gene induction by Hh signaling include the activation of *lethal of scute* (*l'sc*) in the anterior midline daughter cells of the fly embryo. This event leads to the specification of ventral unpaired median neurons and the median neuroblast (Bossing, 2006). A requirement for Hh in the development of specific neuroblast lineages has also been reported (McDonald and Doe, 1997). McDonald and Doe show that *huckebein* expression in rows 1/2 of the neuroectoderm

depends on Hh activity. Despite these individual studies, it is still not clear what other information in addition to Ci activation is required to direct activation of one set of Hh targets vs. another. For example, how is it that Hh induces the expression of *rhomboid* (*rho*) and *stripe* (*sr*) in the ectoderm for proper segmental patterning (Alexandre et al., 1999) while the same source of secreted ectodermal Hh is required for activation of *bagpipe* in the underlying mesoderm (Azpiazu et al., 1996; Bilder and Scott, 1998)? The answer is likely to be the existence of tissue specific factors that collaborate locally with Ci activator to positively regulate gene expression. An example of Ci and cooperative activation has been identified at the *dpp* heldout enhancer that drives *dpp* expression in wing discs (Hepker et al., 1999). Ci in conjunction with the wing "selector" protein Vestigial, are both required for the full *dpp* pattern. For the most part, however, the mechanisms that determine tissue specific activation of Hh targets remain a mystery.

My work has addressed some of the key issues relevant to the mechanism by which Ci regulates the expression of Hh target genes. In the sections of the Discussion that follow, I describe the beginnings of my work, which included a screen for regions in the Drosophila genome that are recognized by the Ci activator and Ci repressor forms. These experiments attempted to identify genes that are directly regulated by Ci as well as to generate an estimate of the landscape of Ci binding regions and their association with specific genes. This screen was complemented by expression array studies to identify genes that change their expression levels in response to Hh signaling. The excellent correspondence between the genes and regions identified by these two independent methods allowed me to identify a high confidence list of putative target genes that I then

subjected to further characterizations. I established that most of the identified putative target genes function and are expressed in a tissue-specific fashion. In addition, I found evidence suggesting that not all components of the Hh signaling pathway regulate the same genes.

Finally, I tested the extent to which Ci activation is capable of up regulating tissue specific targets. I chose genes from three distinct tissue types. The combined results revealed that tissue specific targets are generally capable of Ci mediated up-regulation in a subset of cells that comprise the whole tissue. Because Ci activation was provided ubiquitously and the response was specific to the tissue and location, I suggest that Ci mediated gene activation must be coordinated with local factors.

Overlapping Ci activator and Ci repressor binding regions

Previous reports suggest that activator and repressor forms of Ci function through common Gli binding sites and that a generic consensus binding site confers transcriptional regulation by both forms depending solely on the context of the enhancer (Muller and Basler, 2000). Here, I utilize Ci directed DNA Adenine Methylation (DAMID) to screen for Ci repressor and Ci activator binding regions in the context of the *Drosophila* embryo. The genomic regions identified by these DAMID experiments are associated with Hh targets that are known to be activated in the embryo but also include genes that are specifically activated in larval tissues. Based on the number of genomic regions identified by the DAMCiAct experiment (2438) and the fact that all known

targets are represented by DAMCiAct signal, this is a faithful representation of where Ci can bind in the genome.

I find that Ci repressor binding regions overlap with activator binding regions 889 times, roughly 51% of the total number of Ci repressor binding regions. It is common for several DAMCiRep regions to be found within one CiAct binding region, perhaps reflecting the much larger median width of the DAMCiAct binding regions. 729 CiAct binding regions overlap with at least one CiRep region, suggesting that both activator and repressor forms recognize the same sequences *in vivo*. If both forms of Ci occupied the same binding region, a plausible scenario for Hh induced gene activation could be a transcriptional switch that alternates between a state of default repression and activation. In one event, Ci activator displaces CiRep and the target gene is relieved of repression and receives an activation cue. This scenario may hold true for activation of genes that require high levels of Hh signaling. For example, Ci loss of function clones in the anterior compartment of wing discs are not sufficient to activate *ptc* expression, suggesting that loss of Ci repressor function is not sufficient to activate *ptc* expression. Furthermore, loss of Ci in the *ptc* expression domain results in cell autonomous loss of *ptc* expression, suggesting a need for an activation cue (Methot and Basler, 1999).

The consensus binding site for Gli/Ci, is TGGGTGGTC (Kinzler and Vogelstein, 1990). The rate of occurrence for this motif was determined for DAMCiAct and DAMCiRep binding regions and compared to the rate of occurrence in the entire genome. I find that this motif is significantly enriched within binding regions for both DAMCiAct

(2.55 fold) and CiRep (2.44 fold), providing additional evidence that Ci mediated repression and activation can be carried out by common sequences. However, many CiAct and CiRep binding regions do not overlap, raising the possibility that sequence specific motifs exist for Ci activator and repressor forms. Although the zinc finger DNA binding domain is the same for both forms of Ci, the truncated Ci76 repressor protein could adopt a different folding conformation that allows it to identify different target sequences by interacting with different co-factors. Consistent with this idea, Ci76 repressor is processed from full-length Ci and lacks the binding region that interacts with CBP, a required co-factor that complexes with full-length Ci for *wingless* and *ptc* activation (Akimaru, 1997).

Identification of Hh signaling targets with expression arrays

I made 3 systematic comparisons of the array data based on known observations of the Hh signaling pathway. By applying a clustering algorithm to the array data, I have identified genes that are positively regulated by different Hh signaling pathway members. They group into the following categories: 1) genes that are up regulated when Hh and Smo are present in the embryo, 2) genes that are up regulated when Hh signaling is elevated as in Da Gal4;UAS CiAct embryos and *ptc*- embryos, and 3) genes that are up regulated in wt with respect to individual mutations in *hh*, *smo*, *ci*, and *ptc*. This method produced a total of 199 non-redundant Hh target genes. Among the 199 target genes, I observe DAMCiAct binding regions at a rate of 45% (50% increase over chance). The

high rate of overlap between genes associated with Ci binding regions and transcriptional up-regulation due to Hh signaling provides a list of high confidence target genes.

Regulation of gene targets by different components of the Hh signaling pathway

Genetic and biochemical studies have revealed that Hh functions to activate Smo through an inhibition of Ptc activity (Chen and Struhl, 1996; Taipale et al., 2002). Consistent with these reports, I find genes that positively respond to Hh also respond to Smo, an indication that the sole purpose of Hh is to activate Smo (See above; Figure 6). From the arrays and subsequent analysis, it is apparent that many of these genes are tissue specific targets of Hh signaling (see below). Interestingly, a sub-cluster of genes up regulated in Smo+ embryos do not show a similar response to Hh+ embryos (Figure 6, top sub-cluster). Many of these genes encode proteins that function in general translation and other housekeeping duties. However, two notable exceptions may represent a Smo sufficient mechanism for regulating tissue specific gene regulation and may represent instances where Hh signaling actively down regulates gene expression. First, *dally-like* (*dlp*) encodes a heparan sulfate proteoglycan required for the transduction of the Hh signal (Desbordes and Sanson, 2003; Gallet et al., 2008) and appears to be up regulated in Smo+ embryos and actually *down regulated* in Hh+ embryos (Figure 6). This finding is difficult to reconcile in light of work that shows Dlp to be required for maximal Hh

signaling (Gallet et al., 2008). It is possible that Smo activity sets a permissive condition that, through Dlp activation, allows the initial Hh response. Then, negative regulation by Hh signaling could keep Dlp at appropriate levels once the response has been made. Curiously, there is a precident for Hh signaling in attenuating gene expression of the ladybird genes in heart development (Jagla et al., 1997) and Serrate in epidermal pattering (Alexandre et al., 1999). The other interesting Smo specific target appears to be *suppressor of hairy wing*, (*su*(*Hw*)), a transcriptional repressor that binds to DNA elements called insulators that prevent the activity of local enhancers from driving gene expression at nearby promoters (Cai and Shen, 2001). In cells not receiving the Hh signal, Smo may activate *su*(*Hw*) as a mechanism to keep transcription off. In cells where Hh is received, *su*(*Hw*) is actively repressed, lifting negative regulation of enhancers by insulator elements, allowing transcription to commence. I do not observe DAMID signal at the su(Hw) locus, indicating that this is a Ci independent mechanism.

Loss of Ptc activity in the embryo leads of expansion of Hh target gene expression (Ingham and Hidalgo, 1993). The observed expansion is limited, reflecting the range of de-repressed Smo activity. I made a comparison of transcriptional profiles between loss of function Ptc embryos and ubiquitous gain of function Ci activator embryos to determine if the effect of constitutively activating Smo was similar to activating Ci in all tissues of the embryo on a transcriptional level (Figure 7). The response of genes in embryos associated with loss of Ptc activity or constitutive Ci activator is very similar in terms of the affected genes and the levels to which they respond. Because of the limited nature of tissue specific gene expansion previously seen in *ptc*- embryos and the similar

response to ubiquitous Ci activation on arrays, I conclude that the response to Ci activator in all cells is probably a local response and reflects tissue specific up regulation of gene expression. In accordance with this conclusion is the identification of *drm, drl, sna, and ci* whose levels respond similarly when challenged with ubiquitous CiAct or loss of function Ptc scenarios and whose expanded expression patterns are specific to those tissues in which they are normally expressed (see Figures 9 (*drm*), 12 (*drl*), 12 (*sna*), and 13 (*ci*)).

Ci and tissue specific regulation of gene activation

Classification of genes associated with DAMCi signal and subsequent *in situ* hybridization experiments revealed that many putative Ci targets function and are expressed in distinct tissue types (Figures 3, 4, and 5). This is not surprising given the range of tissue specific phenotypes associated with loss of Hh signaling. However, these results do call in to question how a secreted factor like Hh can activate gene expression in the ectoderm for segmental patterning (Alexandre, 1999) and simultaneously influence pattern in the underlying mesoderm through a different set of targets (Azpiazu et al., 1996). I have taken the results from the DAMID and expression array experiments and identified genes that show a response to Hh signaling in three distinct tissue types: the dorsal ectoderm, the embryonic visual system, and the developing tracheal system.

Dorsal ectoderm

Hh expression in the dorsal ectoderm (DE) is contiguous with expression in the ventral ectoderm, yet three members of the odd skipped family of genes: *odd skipped* (*odd*), *drumstick* (*drm*), and *sister of lines and bowl* (*sob*) are expressed in stripes mainly in cells in and leading up to the edge of the DE. *drm*, *odd*, and *sob* gene expression is missing in *hh* null embryos (not shown) and accordingly, levels of *drm*, *odd*, and *sob* are elevated in wild type embryos with respect to *hh* and *smo* null embryos (Figure 6). The response to ubiquitously expressed CiAct results in expansion of *drm*, *odd*, and *sob* expression in the posterior direction. This effect is specific to the DE and a small spot of cells near the edge of the ventral ectoderm (not shown). The expansion in the DE is limited to approximately 4 cell diameters (Figure 9 F-H), a subset of the cells that normally express *ci* (Figure 10A) and that are capable of inducing *ptc* (Figure 10E). Together, these data indicate that response to CiAct is mediated at the level of the individual enhancer and most likely includes the input of tissue specific factor(s) that allow expansion of *ptc* throughout the segment and expansion of expansion of *drm*, *odd*, and *sob* in a subset of those cells.

Tracheal placode

The results in Figure 10 describe the normal activation of a known Hh target (*ptc*) and a novel Hh target (*ImpL2*) in the developing trachea. Interestingly, at this stage *ptc* expression constitutes a subset of the cells capable of responding to Hh (compare 10B with C). *Impl2* expression is also limited to portions of the tracheal placode that stabilize full length Ci (10D). Expansion of *ImpL2* expression in *Da*>CiAct embryos is an

extension of high level expression found in the dorsal most cells of the placode and includes new expression on the posterior side of the placode(10E). It is interesting to note that an up regulation of *ImpL2* expression occurs in the dorsal ectoderm in a stripe two to three cells wide 10E arrow). *ImpL2* ectopic expression most likely constitutes a subset of the developing trachea. If CiAct is driven in all cells of the tracheal system, a similar albeit smaller expansion of *ImpL2* is observed (10D) and resolves to distinct elements of the tracheal pattern (inset). Because the ectopic activation of *ImpL2* by ubiquitous CiAct occurs specifically in sub-populations of cells in the trachea, I suggest that the effect is due to overlap of activated Ci with trachea specific factors that lead to activation. These putative Ci co-factors would then have to be normally expressed in the sub populations of cells where I observe the effect. Another possibility is that more ubiquitously expressed co-factors overlap with CiAct and tracheal specific factors that generate the pattern I see in 10E. And finally, it is possible that *ImpL2* expression is sensitive to regulation from a dorsal ectoderm enhancer that is normally silent in wild type, but when activated by ubiquitous CiAct, merges with expression driven from the tracheal element.

Embryonic visual primordia

A discussion of Hh signaling in the embryonic visual system starts with an analysis of the expression of known Hh pathway components and targets. A previous report identified *hh* expression in the posterior optic lobe (OLP) and *ptc* expression in the anterior optic lobe (OLA) (Chang et al., 2001). I have extended this analysis to include *smo*, *ci*, *rdx*, and Ci protein (Figure 11). I confirm that *hh* is expressed in the OLP, albeit

at higher levels that previously reported (Figure 11 A arrow). Consistent with the previous report, I find that *ptc* at high levels is largely excluded from the OLP (Figure 11D arrow) and resides in the OLA, in agreement with the dogma that Hh is normally secreted from a source to activate gene expression non-cell autonomously. I find *ci* and *smo* RNA in both lips of the optic lobe primordia with more pronounced *smo* expression in the OLP (11B and C arrows). Curiously, I find evidence that Hh is capable of signaling cell-autonomously in the OLP. The Ci transcriptional target, *rdx*, is present at high levels exclusively in the OLP (11F) arrow. In accordance with Hh target gene activation, I find that full length Ci stabilization occurs almost exclusively in the OLP (11E). Not surprisingly, *en* expression is not present in the OL (Gallitano-Mendel and Finkelstein, 1997; not shown), and thus *ci* expression in the OL is allowed to perdure. The identification of Hh targets and Ci stabilization in the OLP would seem to require some level of Ptc to be present in order to receive the Hh signal. It is possible that Ptc protein is present in the OLP at undetectable yet functional levels. This is consistent with loss of function *ptc* phenotypes that have an effect on Hh target genes in the visual system (see below). The other possibility is that the visual system has evolved a Ptc and perhaps Hh independent mechanism that functions through constitutive Smo activation in the absence of Ptc to activate downstream target genes.

Hh signaling has been shown to play a role in Bolwig's organ development, a derivative of the OLP (Suzuki and Saito, 2000), and mis-expression of Hh signaling causes ectopic eye tissue in the dorso-medial part of the embryonic head ectoderm (Chang et al., 2001). This phenotype was described as cyclopia, the fusion of normally separated bilateral eye tissue. In the case of Hh mis-regulation, anti-22C10 and anti-FasII

staining revealed the ectopic tissue to be Bolwig's organ as well as optic lobe tissue, consistent with the role of Hh in eye development. In this study, I report a similar phenotype that occurs as the result of ubiquitous CiAct expression. One gene, *eyes absent* (*eya*), is required for Bolwig's organ formation (larval eye) and is expressed in the OLP (Suzuki and Saito, 2000) (Figure 12 B arrow). A previous report showed that *eya* expression is controlled by Dpp signaling and that although mis-regulation of Hh and Dpp signaling pathways can lead to a cyclops phenotype, the two pathways do not cross-regulate (Chang et al., 2001). Here, I report a late (stage 11-12) *eya* response to activated Hh signaling in head dorsal mesoderm at a stage when *eya* expression is not present (Figure 12B''). Similar phenotypes occur for *derailed*, *anterior open*, and *snail* (12A'',C'', and D''). The phenotype is consistent with over production of Bolwig's organ precursor cells as these optic lobe markers are all normally expressed in the OLP (12 A-D arrows). Normal expression of these optic lobe genes is not affected in either lip of the optic lobe primordia by mis-expression of CiAct (12 A'-D'). Not shown is the response of *derailed* in *ptc* null embryos which exactly phenocopies the Da>UAS CiAct result. Based on these observations, I conclude that the ectopic gene expression seen in the visual primordia of DA>CiAct embryos is *ptc* dependent and is likely to be a result of direct gene activation by CiAct in the head dorsal mesoderm. The ectopic stripes of gene expression are induced in different widths and at different times, implying that the genes are responding individually and not as a result of one general event. In addition, for these genes, the only ectopic expression I observe in response to ubiquitous CiAct occurs in the developing visual system, which argues for the presence of tissue specific factors that mediate the response to Ci.

ci is a Hh target

A previous report showed that Gli1 not only mediates Hh signaling, but is also a target (Lee et al., 1997). Here, I report a variety of evidence that the same type of regulation occurs in flies. In germ band extended embryos, *ci* RNA is expressed in the ectoderm at homogeneous levels except where *en* represses its activation (Eaton and Kornberg, 1990). In older embryos, perhaps late stage 11 or early stage 12, the expression of ci in the ectoderm becomes graded (Figure 13B). Higher levels of ci RNA are clearly present in cells which receive the Hh signal, leading me to test whether constitutive Hh signaling is capable of activating *ci*. Indeed, ubiquitous CiAct causes cells posterior to the Hh signaling domain to express elevated levels of *ci* (13D). What could be the biological role of this positive feedback loop? The accumulation of ci RNA in Hh receiving cells is certainly consistent with activation of other Hh targets in a uni-directional manner. Perhaps the activation of *ci* ensures a sufficient pool of full length Ci protein template is present for conversion into a transcriptional activator.

Experimental Procedures

Fly strains, *in situ* hybridization and immunostaining

Embryos for array analysis

To generate null homozygous mutant embryos lacking Ptc or Hh activity, heterozygous null alleles of *hh* and *ptc*: hh^{13C}, hh^{AC}, ptc^{B98}, and ptc^{13C} over green balancers (TKG4 and CKG19; Casso et al., 2000) were crossed at 25ºC to generate hh^{13C}/ hh^{AC} and ptc^{B98}/ptc^{13C} embryos and scored by virtue of missing GFP. Trans-heterozygous sibling embryos from both crosses (1 copy of GFP) were kept as hybridization controls. To generate *ci* null embryos, a stock harboring a *ci* rescue construct ci^{RES} , was balanced over a GFP balancer (Casso et al., 2000) in a *ci* null background: ci^{RES}/GFP; ci^{94}/ci^{94} (Brenda Ng, unpublished stock). Embryos homozygous for GFP were scored as *ci* null embryos.

To eliminate the maternal and zygotic contributions of *smo*, Smoothened null germline clones were generated using the Ovo^{D1}dominant female sterile technique (Chou and Perrimon, 1996) and the following crossing scheme: virgin females +/+; smo^{Q},FRT40A/Cyo were crossed to males yw, HsFlp/Y; *sco*/Cyo. From this cross I collected virgin females w,HsFlp/+; smo^{Q}, FRT40A/Cyo and crossed to males +/Y; OvoD1,FRT40A/Cyo. The embryonic and first instar larvae progeny were heat shocked in a 37ºC bath for 1 hr. every day until the flies eclosed. The only fertile female progeny will have undergone a recombination in the germ cells to produce a *smo* null germ line. These females were crossed to males smo^{Q} /CKG. From this cross embryos were

collected and scored against GFP to isolate *smo* null embryos. Embryonic cuticles were examined to confirm loss of *smo* activity.

Gal4;UAS crosses

All Gal4;UAS crosses were incubated at 25ºC in a humidified incubator.

Stocks: *Da* and *btl* Gal4 was obtained from the Bloomington stock center, Bloomington, Indiana. UAS Cim1-m4 (UAS CiAct) was provided by S. Smolik.

In situ hybridization and immunostaining

Dig-labeled anti-sense RNA probes were synthesized and hybridized to whole-mount embryos according to O'Neill and Bier (1994). Embryos were incubated with Rat anti-2A1 antibody (Ci^{Fl}) at 1:1000, labeled with an anti-Rat biotinylated secondary antibody at 1:500 (Vector) and signal was visualized with the ABC vectastain kit (Vector laboratories). Stained embryos were mounted in Permount.

Microscopy

All images of embryos or wing discs were taken on a compound DMR Leica microscope using 40x, 63x, and 100x objectives under Nomarski optics.

Constructs

N terminally fused DAMID constructs were generated by PCR amplifying BglII-XbaI fragments of Ci76 (Aza-Blanc et al.,1997) and Ci m1-m4 (template kindly provided by S. Smolik) using the forward primer:

5'TAAGATCTTATGGACGCCTACGCGTTACCTAC and reverse primers: 5' TAATCTAGAGTCTGCCACGTCCACGTCATCGT for Ci76 and

5'TAATCTAGACTGCATCATTTGAAGGTATCTATTTTCC for Ci m1-m4. The PCR

products were then digested with BglII and XbaI and ligated to BglII, XbaI digested pNDamMyc (provided by Bas van Steensel). The resulting DamCi fusion cassettes were excised by partial digest with EcoRI, XbaI and subcloned into pUAST.

DAMID

Probe synthesis

Digestion and PCR amplification of DAM-methylated DNA was done as previously described by Choksi et al., 2006. Whole embryos from DamCi or Dam alone flies were collected after 2 hours and aged for an additional 4 hours at 25°C. For selective PCR amplication of methylated DNA fragments, 2.5 μg of the isolated genomic DNA was digested for 16 hr at 37°C with ten units DpnI (NEB) in a total volume of 10 μl buffer 4 (NEB). After inactivation of DpnI at 80°C for 20 min, 1.25 μg of the DpnI digested genomic DNA was ligated to 40 pmol of a double-stranded unphosphorylated adaptor (top strand, 5'CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA-3'; bottom strand, 5' TCCTCGGCCG-3' 2) for 2 hr at 16°C with five units T4-DNA Ligase (Roche) in a total volume of 20 μl ligation buffer (Roche). To prevent amplification of DNA fragments containing unmethylated GATCs, the adaptor-ligated DNA was cut with five units DpnII (NEB) for 1 hr at 37°C in a total volume of 80 μl DpnII buffer (NEB). Next, amplification was performed with 20 μl DpnII-cut DNA (313 ng), 1.6 μl PCR Advantage enzyme mix (Clontech), 16 nmole of each dATP, dCTP,

dGTP, dTTP, and 100 pmole primer (5'GGTCGCGGCCGAGGATC-3') in 80 μl total volume of PCR Advantage reaction buffer under the following cycling conditions: activation of the polymerase and nick translation for 10 min at 68°C, followed by one cycle of 1 min at 94°C, 5 min at 65°C, and 15 min at 68°C; three cycles of 1 min at 94°C, 1 min at 65°C, and 10 min at 68°C; and 19 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 68°C. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

Tiling arrays and hybrizdization

To map Ci binding sites on a genome-wide scale, a custom whole genome 375,000 feature tiling array, with 60-mer oligonucleotides spaced at approximately 300 bp intervals, was designed against Release 4.0 of the Drosophila genome (Choksi et al., 2006). The control (Dam alone embryos; flies provided by S. Parkhurst) and experimental samples (DamCiAct or DamCiRep) were labeled and hybridized to these custom arrays. Arrays were then scanned, and intensities extracted (Nimblegen Systems).

DAMID Analysis (K. Kechris)

Normalization

At each tiling array feature, accounting for the dye swap replicate, the log ratios between the target and control sample were calculated using the *limma* package in R (Smyth et al., 2005). The data were then normalized with *limma*, applying the ``loess'' option for normalization within arrays and ``Aquantile'' option for normalization between arrays.

Obtaining binding regions

To predict binding regions, we first performed an analysis at the feature level and then examined sliding windows of features across each chromosome. In the first step, each feature was tested for significant intensity using a one-sided t-test. Since the sample size (n=3) is small, a method implemented in *limma* was applied that pools information from all features to obtain more stable variance estimates for the t-statistic. In the second step, p-values for consecutive features were "averaged" using a meta-analysis approach to identify genomic regions of high signal intensity (details in Kechris et al., Submitted). In particular, for each feature, a window size of *w* features is considered on each side of the feature. An "averaged" p-value is calculated on the *2*w+1* p-values in the *w*-neighborhood for that feature correcting for local dependencies among features. Because of the large number of significance tests, p-values were corrected for false discovery rate (FDR) control using the Benjamini & Hochberg procedure (Benjamini, 1995). For both CiRep and CiAct *w* was set to 4.

Binding regions were created by scanning features in order of each chromosome. If a feature had adjusted p-value below some set FDR cutoff a new binding region was formed. The next feature passing the cutoff in the linear chromosome order was then evaluated to see if it is within *w* features and 700 bp of the last feature in a binding region. If so, it was added to that binding region, otherwise a new binding region was created. This procedure was continued in a step-wise fashion until the last feature on the chromosome was evaluated. The FDR cutoff was set to .02 and .001 for CiRep and CiAct

respectively. The cutoff was more stringent for CiAct to reduce the number of binding region predictions since the signal was much stronger in CiAct.

Ci binding motif enrichment

Enrichment of TGGGTGGTC in binding regions was evaluated using scripts written in the perl v5.10.0 scripting language. Based on the genomic coordinates for each binding region, binding region sequences were extracted from the genome sequence files obtained from FlyBase Release4 (Wilson et al., 2008). A perl script was then used to count the occurrences of the TGGGTGGTC motif, including its reverse complement, within the two sets of binding region sequences and the entire genome. The rate of motif occurrences in the binding region sets and the genome was calculated by dividing the counts of the observed motif by the total length of the binding region sequences or the genome respectively. Enrichment of the motif was defined as the rate of the motif occurrences in the binding region set divided by the rate in the genome. For CiRep, enrichment was 2.42 (p-value = 7.2e-09) and for CiAct, enrichment was 2.55 (p-value = 4.0e-24). P-values were obtained assuming a Poisson distribution for the motif counts.

Overlap of CiAct with CiRep binding regions and ranking of CiAct-CiRep overlap

Genomic positions of CiAct and CiRep binding regions were compared to identify the number of times that binding regions from the two different experiments overlapped by at least 1 base pair. Of the 1743 CiRep binding regions, 889 overlapped with CiAct binding regions (~51%). Of the 2438 CiAct binding regions, 729 overlapped with CiRep binding regions (~30%). A relatively larger percentage of CiRep binding regions overlap CiAct

binding regions because they tend to be shorter, so multiple binding regions may overlap with a single CiAct binding region. The 729 CiAct binding regions that overlapped CiRep binding regions were sorted by the minimum feature p-value in the binding region or equivalently the maximum $-\log_{10}$(p-value).

Alternatively, using the v4.0 FlyBase collection of genes (Wilson et al., 2008), we examined the overlap of genes neighboring the two sets of binding regions. Using the genomic coordinates of the binding regions and genes from FlyBase, "neighboring genes" are defined as the closest upstream and downstream gene for each binding region. Genes appearing multiple times for different binding regions in a set were only counted once. 1825 of the neighboring genes overlap, ~66% of the 2747 CiRep neighboring genes and ~52% of the 3521 CiAct neighboring genes.

Overlap of CiAct binding regions with expression "targets"

As defined above, neighboring genes for the CiAct binding regions were compared to a set of genes determined by a series of gene expression experiments to be likely targets of Ci called ``target genes". Target genes were identified by having to show a median gene expression fold induction of >1.4 in the following comparisons: wildtype vs. null genetic backgrounds for *ci*, *ptc*, *hh*, and *smo* and DaGal4;UAS CiAct vs. wildtype. Only the 199 target genes that also had annotation in the v4.0 FlyBase collection were retained. Enrichment of target genes was defined as the percentage of target genes overlapping the neighboring genes divided by the percentage of neighboring genes in the genome. For

example, if one set of binding regions has 2695 neighboring genes (~20% of the 13472 total genes), which intersect with 60 target genes, (~30% of the 199 target genes), then target gene enrichment is ~.3/.2 = 1.5, indicating that 1.5 more target genes were in the overlapping set than expected by chance.

Microarray

RNA amplification

Total RNA isolated from embryos was incubated with 10 ng oligo(dT)24 -T7 primer (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCG-GTTTTTTTTTTTTTTTTTTTTTTT) and 125 ng TS primer (AAGCAGTGGTAACAACGCAGAGTACGCGGG) in 5 µl at 65˚C for 10 min and cooled on ice. Six microliters first-strand premix were added and incubated at 42˚C for 2 h (6 µl mix was made up with 2 µl 5x first-strand buffer, 1 µl 0.1 M DTT, 1 µl dNTPs (10 mM each), 0.3 µl T4gp32 (USB, 13.82 mg/ml), 0.5 µl RNasin (Promega), 1 µl Superscript II (Invitrogen)). The reaction was incubated at 65˚C for 10 min and cooled on ice. Cold second-strand premix (64.5 µl prepared on ice: 45 µl RNase-free water, 15 µl 5x second-strand buffer (Invitrogen), 1.5 µl dNTPs (10 mM each), 0.5 µl E. coli ligase (10 U/µl), 2 µl E. coli polymerase (10 U/µl), 0.5 µl E. coli RNaseH (2 U/µl)). Enzymes for the second-strand premix (Invitrogen) were added and incubated at 16˚C for 2 h, followed by the addition of 2 units T4 DNA polymerase (Promega) and incubation at 16˚C for 15 min before heat inactivation at 70˚C for 10 min. Clean-up was performed

with DNA clean and concentrator-5 (Zymo Research), eluting twice with 8 µl RNase-free water. The total volume was adjusted to 8 µl in a Speed Vac. IVT was performed with Megascript T7 (Ambion) in a 20 µl volume for 5-6 h, followed by DNA digestion. aRNA was purified using Mini RNA Isolation Kit or RNA Clean-up kit (Zymo Research), eluting in 2 x 8 µl RNase-free water. Hybridization probes were labeled by incorporation of amino-allyl modified nucleotides in a first-strand cDNA RT reaction. Monofunctional Cy5 or Cy3 dye (Amersham) was subsequently coupled to the reactive residues. Multiple hybridizations were carried out, and dye labeling was reversed to avoid systematic bias.

Data analysis

Hybridized microarrays were scanned with a GenePix 4000 A Microarray Scanner (Axon Instruments, Union City, CA). Data were analyzed and displayed with Cluster and Treeview (Eisen et al., 1998), Genepix PRO (Axon Instruments), and Microsoft Excel. Normalization for cluster analysis was done with NOMAD 2.0. Only genes that qualified with a combined median intensity > 300 above background in both channels in at least 80% of the repeated experiments were included in the analysis. A threshold of >1.4 (=.5 of the log2-transformed ratios) was chosen for all comparisons.

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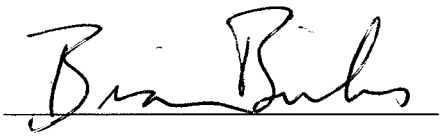

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