A dsRNA Based Screen Identifies Novel Proteins Involved in Drosophila Hedgehog Signaling Pathway

Senior Honors Thesis, Northwestern University

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I. Abstract

A screen has been performed identifying potential novel members of the Hedgehog (Hh) signaling pathway. The Hh signaling pathway is responsible for the patterning of many different organs during development. In mammals, aberrant Hh signaling is associated with a number of different cancers, including basal cell carcinoma and prostate cancer, and therefore, a further understanding of Hh could lead to new treatments. While many proteins involved in the Hh signaling pathway have been discovered, there are still many that remain unknown.

In *Drosophila melanogaster*, the Hh signaling pathway is responsible for the patterning of the wing, so a UAS-Gal4 RNAi screen was devised using the MS1096-GAL4 driver to specifically knock down gene expression in the wing. The screen was performed in a sensitized *fused* (*fu*^{1}) mutant background, looking for both suppressors and enhancers of the *fu*^{1}phenotype. By observing wing vein patterning, it was possible to determine whether loss of a particular gene had a significant effect on Hh signaling. Thus far, the screen has covered 11% of the *Drosophila* genome. Out of 1590 genes, 29 were strong enhancers and 11 were suppressors of the *fu*^{1} phenotype. Several of these hits were followed up using RNAi in larval wing discs and visualizing gene expression with immunofluorescence. One gene of particular interest, *megator* (mtor), was a strong enhancer of the *fu*^{1} phenotype. Knockdown of *mtor* with RNAi resulted in a decrease in expression of the Hh target genes *decapentapleigic* and *collier*. Future research will include looking for genetic interactions with a mutation in the endogenous *mtor* gene to validate its involvement, and elucidating the function of Mtor in the Hh signaling pathway.

II. Introduction and Literature Survey

A: Introduction

Life begins as a single cell which divides to produce the ten trillion cells in the human body. Each of these cells must differentiate in order to perform a specific role in the overall functioning of an adult organism. The development of that single cell is carefully controlled to ensure that all of the necessary components form in the correct place and time. In a multicellular organism, each cell is not a separate entity but must function in concert with its neighbors. In order for proper development to occur, cells must be able to communicate with one another, which they do through the use of signaling pathways.

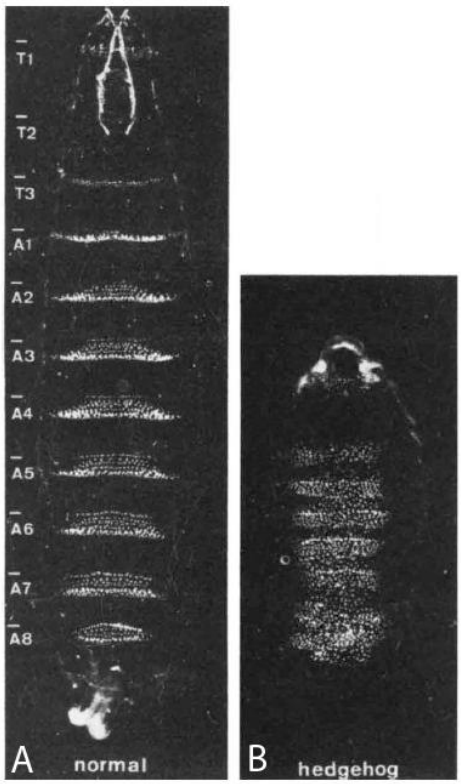


Figure 1. Hh mutant Larva display a distinct phenotype.
A: *Drosophila melanogaster* larva, wild-type. B: Larva mutant for *hh*. Obtained from Nusslein-Volhard et al. (1980)

Signal transduction pathways are a means by which a cell can receive exterior signals, often produced by other cells, and then internalize that message and initiate a response. One such signaling pathway that plays a crucial role in the development of everything from flies to humans is the Hedgehog (Hh) signaling pathway. Nusslein-Volhard and Wieschaus first discovered the Hh signaling pathway in 1980, when searching for mutants in *Drosophila melanogaster* that caused errors in larval segmentation. Normally, *Drosophila* larvae are comprised of eleven body segments which are anteriorly marked by bands of denticles (Fig 1A). In larva mutant for Hh, it appears that all segment boundaries are missing

(Fig 1B) and the entire body of the larva is covered in denticles - making them look like hedgehogs (Nusslein-Volhard & Weischaus, 1980).

B. Studying Hh Signaling in *Drosophila*

Drosophila are ideal model organisms for studying the Hh signaling pathway. In *Drosophila*, the Hh signaling pathway is not only involved in segmentation, but also the patterning of adult appendages such as the wings and the nervous system. Most of the pathway components are conserved in mammals, though over time some have undergone modifications in function or duplications leading to redundancy (Wilson & Chuang, 2010). These changes make looking for new members of the Hh signaling pathway more difficult and inefficient in a mammalian model. A short generation time and large number of progeny are both useful aspects of *Drosophila* when performing a genome-wide screen for novel members of the Hh signaling pathway, as well as the availability of numerous genetic tools that can be easily used in this model organism.

One key tool is RNA interference (RNAi), which is a method used to prevent the expression of a specific gene. RNAi takes advantage of a cell's natural defense mechanisms against double stranded RNA (dsRNA), which is commonly used by viruses and transposons as an intermediate when invading a host's genome (Carthew, 2001). When dsRNA is found by a cell, it is first processed by Dicer (Dcr), a ribonuclease which cuts the dsRNA into 21-23 nucleotide fragments, called short interfering RNAs (siRNAs) (Fig 2). The two strands of the siRNA are then unwound and one of the strands is loaded into the RNA-induced silencing complex (RISC). This multiprotein complex functions as an endonuclease, cleaving any mRNA that is sufficiently complementary to the siRNA (Sontheimer, 2005). The end result is prevention of unwanted protein production through the targeted degradation of any viral mRNAs.

Fire et al. (1998) discovered that injecting dsRNA into *C. elegans* results in gene silencing, and since then RNAi has been developed as a valuable technique for gene-inactivation. Paddison et al. (2002) showed that endogenously encoded genes can also induce RNAi through the transcription of short hairpin RNA (shRNA) precursors that are cleaved into siRNA. Transgenic flies can now be ordered that contain shRNA that will result in knockdown of almost any particular gene in the *Drosophila* genome.

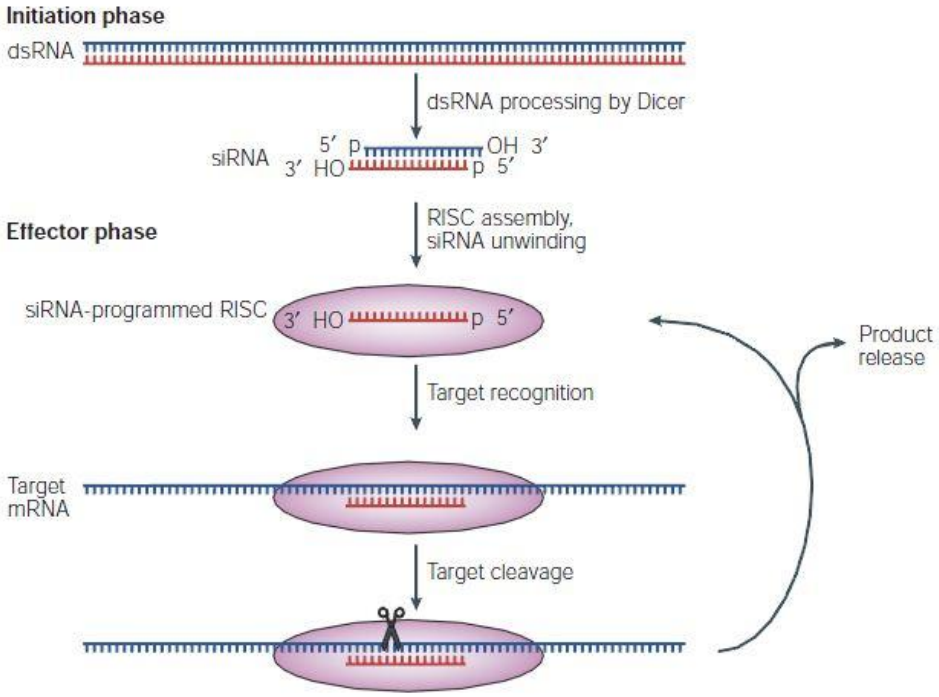


Figure 2. The general pathway of RNAi *in vitro*.
RNAi consists of two phases, initiation and effector. During in initiation, dsRNA is identified and cut by the endonuclease Dicer, resulting in 25 nucleotide siRNA fragments. After unwinding and being loaded into the Risc complex, the single siRNA strand then targets complementary target mRNA sequences, resulting silencing of its targets. Obtained from Sontheimer (2005).

C. An Overview of the Pathway

The larval wing disc, which develops into the adult wing, is divided into several different compartments (Fig. 3A). In regards to the Hh signaling pathway, the most important are the anterior and posterior compartments. Hh is produced by cells in the posterior half of the wing disc, but these cells lack the ability to respond to this signaling molecule. Hh diffuses over to the

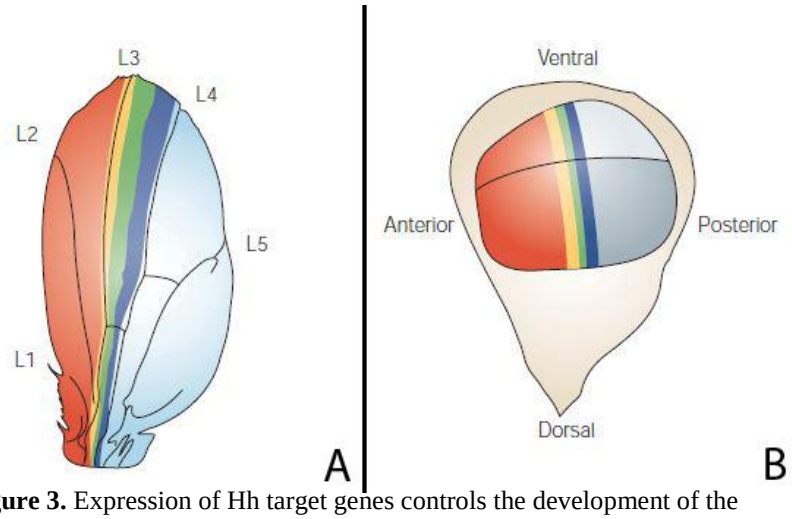


Figure 3. Expression of Hh target genes controls the development of the larval imaginal disc into the adult wing.
A: Adult fly wing. The area between veins L3 and L4 is patterned by the Hh signaling pathway. Target gene expression is show in blue, green, and yellow. B: Larval wing disc. Colors match corresponding structures in developed wing. Hh is produced in the posterior compartment (gray) and diffuses to the anterior, resulting in a graded response in gene expression. Obtained from Hooper and Scott (2005).

anterior compartment, where the cells express the proper cellular machinery to respond and interpret the signal. In the absence of Hh, the transmembrane protein Patched (Ptc) inhibits Smoothened (Smo) by shifting the equilibrium of its trafficking between the cell surface and endosomes, preventing Smo accumulation at the membrane. Binding of Hh to Ptc causes a conformational change which prevents this inhibition of Smo, though the exact mechanism of this inhibition is unknown. The signal is transduced through the cytosol by a signaling complex comprised of Costal2 (Cos2), Fused (Fu), and Suppressor of fused (Su(fu)). This complex is involved in regulation of the transcription factor Cubitus interruptus (Ci). Ci exists either at its full length or as a truncated form that is the result of partial proteolysis, and it is the equilibrium between these two states that is modulated in response to Hh. When Hh is absent, Cos2 associates with microtubules and recruits Protein Kinase A (PKA), Casein Kinase 1 (CK1), and Glycogen Synthase Kinase 3 (GSK3) into a complex that phosphorylates Ci, targeting it to the proteasome. After partial degradation, Ci is then able to enter the nucleus and repress the transcription of Hh target genes. In addition, Su(fu) has been shown to retain full-length Ci in the cytoplasm (Hooper & Scott, 2005).

When Hh is present, disinhibited Smo undergoes conformational changes that allow the cytosolic signaling complex to associate with Smo's cytosolic tail via interactions with Cos2. The recruitment of this complex to Smo results in decreased phosphorylation of Ci and increased phosphorylation of Smo, Fu, Su(fu), and Cos2 (Lum & Beachy, 2004). Phosphorylation of Smo results in its stabilization, leading to a strengthening of the signal induced by Hh, and phosphorylation of Cos2 by Fu is thought to lead to destabilization of the Cos2-Ci complex which helps to prevent the targeting of Ci to the proteasome. Full-length Ci is then able to enter the nucleus and activate gene transcription (Hooper & Scott).

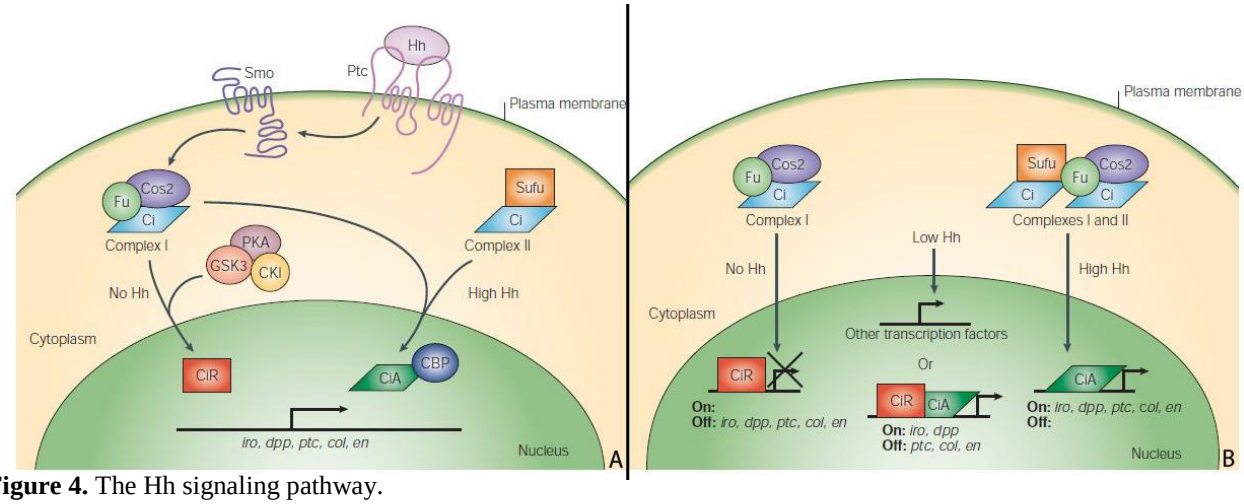


Figure 4. The Hh signaling pathway.
A: When Hh is absent, Ptc inhibits its fellow transmembrane protein, Smo. The Cos2-Fu complex binds Ci, allowing it to be phosphorylated by the kinases PKA, GSK3, and CKI. This phosphorylation targets Ci to be partially degraded by the proteasome. A truncated form of Ci is released, and is then free to either the nucleus where it acts as a repressor of target genes. When Hh is present, it binds to its receptor, Ptc, which disinhibits Smo. Smo recruits Cos2-Fu and its associated kinases, and so Ci is no longer targeted to the proteasome and remains as in its full-length form. Full length Ci then is free to enter the nucleus, along with Sufu, and activates Hh target gene expression.
B: The level of Hh signaling affects target gene expression through the transcription factor Ci. When no Hh is present, Ci is targeted to the proteasome and partially degraded, leading to a truncated repressor form that is able to enter the nucleus and turn of target gene expression. When low amounts of Hh are present, Ci is still partially degraded, but some remains in its full length form. These two forms compete for binding sites, leading to the activation of certain target genes. When high amounts of Hh are present, all Ci escapes proteolysis and enters the nucleus in its full-length, activator form, and is able to turn on all target genes. Obtained from Hooper and Scott (2005).

Regulation of Ci

As a transcription factor, the role of Ci is to regulate gene expression by entering the nucleus. Despite containing a bipartite nuclear localization signal (NLS), full-length Ci is primarily located in the cytoplasm in the presence or absence of the Hh signal. The presence of

robust nuclear export signals, along with associations with cytoplasmic protein complexes, retains Ci in the cytoplasm. Phosphorylation by PKA primes Ci for further phosphorylation by CK1 and GSK3, which signals to E3 ubiquitin ligases to target Ci to the proteasome. After undergoing partial degradation of its C-terminus, Ci is then released from the proteasome. This truncated form of Ci (Ci_{rep}) retains the NLS as well as five zinc finger domains that confer specificity in DNA binding, and is then free to enter the nucleus where it binds to Hh target genes and prevents their expression (Fig 4) (Hooper & Scott).

In the presence of Hh, Ci no longer undergoes partial degradation, due to the recruitment of the Cos2-Fu complex to Smo, along with the three kinases PKA, CK1, and GSK3. Ci remains in its full-length form and then enters the nucleus along with Su(fu) to turn on target gene expression (Sisson et al, 2006). However, lack of proteolysis is not enough to result in a full Hh response. Wang and Holmgren (1999) showed that loss of Cos2 and Fu could not mimic Hh signaling, which suggests that Ci exists in not only truncated-repressor and full-length forms but also as an activated-full-length form (Ci_{act}). The exact mechanism of this activation is still unknown. Once in the nucleus, Ci_{act} then binds to target genes and induces their expression (Fig 4).

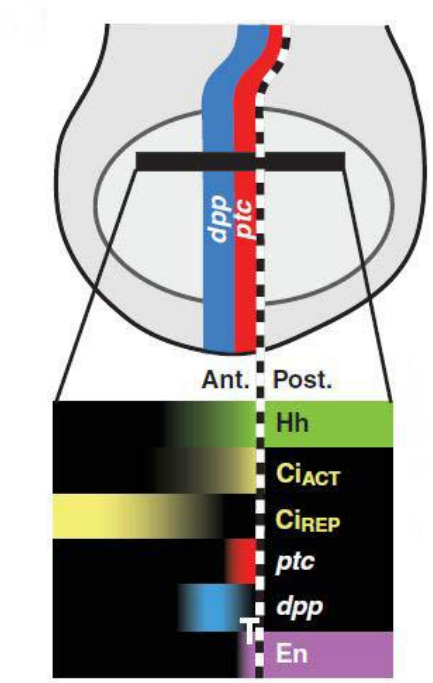


Figure 5. Gradients are responsible for target gene expression.
Hh is produced by cells in the posterior of the wing disc and forms a gradient across the anterior compartment. This leads to the formation of other gradients, namely a high concentration of Ci_{act} near the boundary that is able to turn on target gene expression. Each target gene is turned on at different levels of Ci_{act} and Ci_{rep}, leading to characteristic stripes of gene expression at specific distances from the AP boundary. Obtained from Parker et al (2011).

Control of Target Gene Expression

A number of genes are up-regulated by Ci in response to a signal from Hh. Because Hh is only

produced in the posterior compartment, its diffusion results in the formation of a gradient across the anterior compartment. The highest concentration of anterior compartment Hh is found directly at the AP border and the cells furthest away from this boundary in the anterior compartment essentially receive no Hh. The concentration of Hh that a cell receives determines the ratios of Ci_{act} to Ci_{rep} present in the nucleus, thus affecting the expression of target genes. The cells immediately at the AP boundary receive a high concentration of Hh, which results in the expression of genes such as *engrailed* (en), *collier* (col), and *patched* (ptc). *decapentapleigic* (dpp) responds to moderate levels of Hh, and so it is expressed in a stripe of cells further away from the AP boundary (Fig 5, 6) (Hooper & Scott).

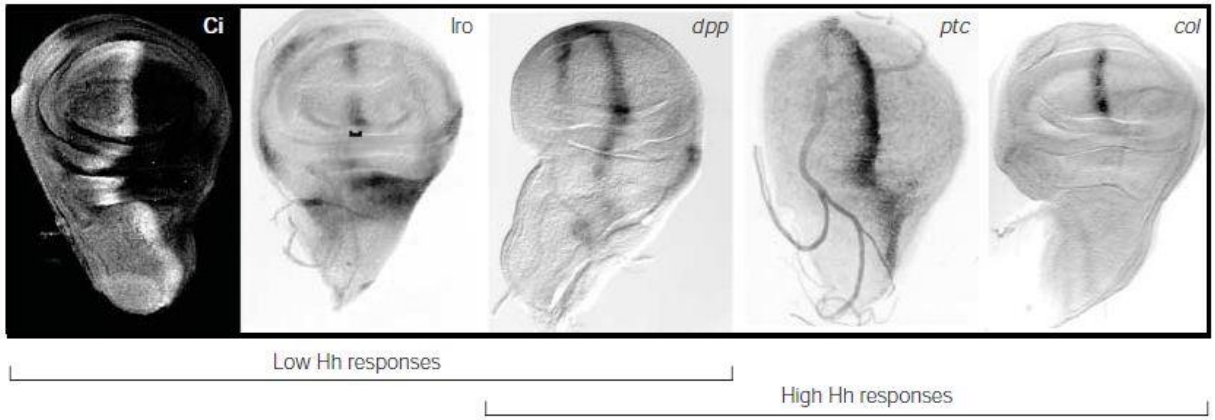


Figure 6. Visualization of Hh target genes shows distinct patterns of expression.
Obtained from Hooper and Scott (2005).

Target gene expression is controlled by competition between Ci_{act} and Ci_{rep} for the same enhancer element binding sites. Barolo et al. (2011) propose a model that relies on both binding site affinity and cooperative repression to explain the localization of target gene expression in the wing disc. Because Ci_{rep} cooperatively interacts with itself, it can outcompete Ci_{act} when binding to high-affinity binding sites which are present in enhancer elements of high-level Hh target genes. In the anterior cells immediately at the AP-border, the high level of Hh results in a greater concentration of Ci_{act} that can occupy high-affinity binding sites, inducing gene expression.

However, in cells further away from the AP border where Ci_{rep} is present along with Ci_{act}, high-affinity binding sites induce repression rather than activation. In these cells, moderate-level Hh target genes rely on low-affinity binding sites for their expression, which combat the cooperative binding of Ci_{rep} and allow Ci_{act} induced gene expression. Overall, these mechanisms determine the spatial organization of Hh target gene expression across the wing disc, which controls its development into an adult wing.

D. Screening for Novel Pathway Components

As mentioned above, Hh was first pulled out during a screen of segment polarity mutants performed by Nusslein-Volhard and Wieschaus (1980). The goal of their screen was to investigate the genes involved in the spatial organization of a developing embryo, in the hopes of better understanding segmental pattern formation.

Since then a number of other screens have been performed to discover the proteins involved in the Hh signaling pathway, followed by further work to determine their function and interactions with the rest of the pathway. In 1988, Perrimon et al. performed a screen of X-linked loci looking for zygotic lethals that exhibited maternal effect phenotypes. They identified *fused*, which encodes for a kinase that was later discovered to have a role in the complex that targets Ci to the proteasome.

Haines et al. (2000) performed a mutagenesis screen for dominant mutations that suppress phenotypes due to the overexpression of Hh in the wing, from which they identified four mutations that they then analyzed with immunohistochemical staining in wing disc. In 2003, Lum et al. performed a screen in *Drosophila* cultured cells, using RNAi in order to assay the functional roles of kinases and phosphatases, and 43% of predicted *Drosophila* genes. They were able to identify Dally-like protein (Dlp) and Caesin Kinase 1 α (CK1α). Dlp was a known cell

surface molecule, which further analysis showed acts upstream or at the level of Ptc in the Hh signaling pathway. Overexpression of CK1α was shown to result in suppression of pathway activation due to Hh, and they suggested that it may play a role in the phosphorylation of Ci.

In 2005, Collins et al. screened for mutations that were able to suppress or enhance a hypomorphic Hh phenotype in the wing, which was the result of C756-Gal4 driven expression of Smo RNAi. They were able to identify new alleles of known pathway components, including *ptc* and *smo*, as well as 105 novel interacting mutations. Nybakken et al. (2005) performed a genome-wide RNAi screen in *Drosophila* cell culture, which identified hundreds of potential new regulators of Hh, some of which had already been implicated in aspects of Hh signaling by previous screens, such as eRF1.

Another enhancer/suppressor screen was performed by Casso et al. (2008), which discovered both novel components of the pathway as well as 26 autosomal regions that interact with Hh. Their screen relied on ptc-gal4 to express smo RNAi, leading to a partial fusion of wing veins 3 and 4. Flies with this phenotype were crossed to flies carrying deletions of various chromosomal regions. This screen resulted in the identification of *microtubule star* (*mts*), which is necessary for full activation of Hh signaling, and *second mitotic wave missing* (*swm*), which was shown to be a negative regulator of the Hh signaling pathway.

E. The Importance of Hh Signaling

As in flies, the vertebrate Hh signaling pathway is involved in many different developmental processes. One well studied role of Hh is in the patterning of the vertebrate limb, where a concentration gradient of Hh is responsible for the determination of digit identity (Saunders, 1968). Hh is also involved in induction of the neural tube, and ultimately is responsible for the development of structures in both the spinal cord and the brain. In adults, the

Hh signaling pathway is mostly quiescent, but has been implicated in promoting the proliferation of many different types of stem cells, including neural and hematopoetic (Bhardwaj et al., 2001; Ahn & Joyner, 2005).

Disruption of the Hh signaling pathway during embryogenesis can lead to drastic developmental abnormalities such as holoprosencephaly, where the forebrain fails to divide into two hemispheres, often resulting in cyclopia (Copp & Greene, 2010). Embryos that do not receive enough Hh signaling may also have underdeveloped limbs. Increased amounts of Hh during development can lead to the growth of too many digits (Rodriguez, et al., 1996).

Aberrant Hh signaling has been implicated in tumor formation and is known to be involved in many different cancers. These include basal cell carcinoma, medulablastoma, prostate cancers, as well as many others. There have been three models proposed for the involvement of the Hh signaling pathway in different types of cancer. The first is Hh independent cancers, which involve a mutation that activates the Hh signaling pathway downstream of Hh. The second type of cancers is those that are Hh dependent, where the same cells both produce and respond to Hh in an autocrine manner. The third type is also Hh dependent, but these tumors act in a paracrine fashion. Another putative model has proposed that the Hh signaling pathway is required for the maintenance of cancer stem cells, which comprise a small subset of an entire tumor and are responsible for producing all the other cancer cell types. It is important to know which model applies to a specific cancer in order to determine the most effective treatment. The Smo inhibitor, which targets the Hh pathway component Smo, is the only Hh pathway inhibitor that has been tested in humans, so far. Some, such as cyclopamine, can be applied as a cream to the skin in order to treat basal cell carcinomas. Newer treatments that are showing great promise are more potent inhibitors that can be taken orally (Scales &

Sauvage, 2009). Hopefully further research into the Hh signaling pathway will lead to not only a better understanding of these diseases but also new methods for treating them.

III. Materials and Methods

A. Drosophila stocks and culture

Stocks containing RNAi were obtained through the Vienna Drosophila RNAi Center and the Drosophila Genetic Resource Center, Kyoto Stock Center. Other stocks were obtained through the Bloomington Stock Center. Flies were cultured on standard cornmeal/molasses medium at 25ºC.

B. Experimental Fly Crosses

Fused^{1} screen. Female flies containing the *fu^{1}* mutation, which results in a fusion of the longitudinal wing veins 3 and 4, and MS1096-GAL4 wing-specific driver on their X chromosome, were crossed to males containing RNAi. The resulting progeny were screened for enhancement or suppression of the *fu^{1}* phenotype.

Imaginal Disc Staining. RNAi-containing flies were crossed to either w *fu^{1}*; AP-GAL4/CyO, yw; Dpp-lacZ, AP-Gal4/CyO y+, or w; Dpp-LacZ, AP-GAL4/CyO; UAS-GFP/Tb. Resulting third instar larvae were dissected and stained using the immunohistochemical procedure described below.

Generation of Somatic Clones. Clones of mutant cells were generated through FLP-mediated recombination (Xu and Rubin, 1993). Clones were created 48-72 hours after egg laying by heat shocking larvae for 60 minutes at 37ºC. Crosses were performed between yw HSFLP; P{neoFRT}42D, P{lacW}Mtor/CyO y+ and either yw HSFLP; P{neoFRT}42D, ubi-GFP/CyO or yw HSFLP; P{neoFRT}42D, P{πM}45F, M(2)53/CyO.

***hh^{mrt}* Interaction**. Interactions with hh^{mrt} were scored as in Casso et al. (2008). The adult wings were mounted on slides and imaged. ImageJ software was used to determine the ratio of the area

between veins 3 and 4 to the total area of the wing. A t test was used to analyze variance between experiments and controls.

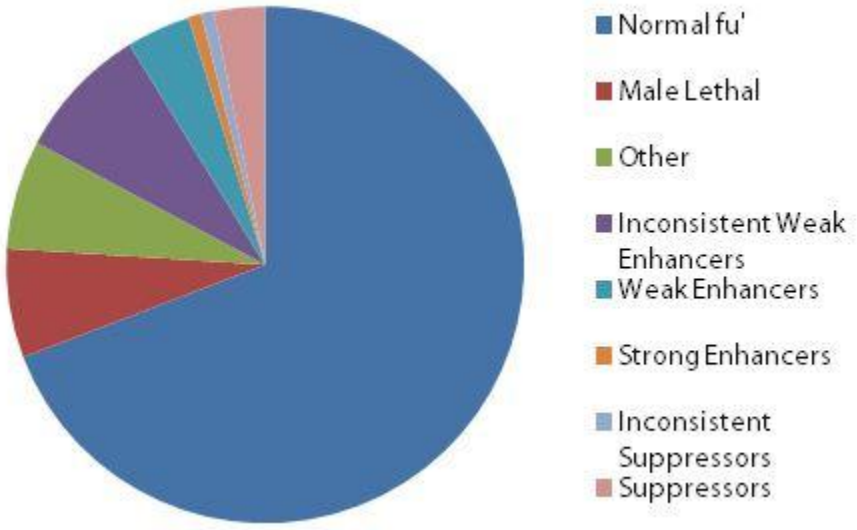
C. Immunohistochemistry

Imaginal discs were prepared as in Carroll and Whyte (1989). Images were collected on a Zeiss Axiophot microscope fitted with a digital camera and analyzed with VayTek deconvolution software. Antibody staining of imaginal discs and salivary glands were performed with anti-β-galactosidase (Buenzow and Holmgren, 1995), antibody 2A1 (Motzny and Holmgren, 1995), antibodies Apa1, 4D9 (Developmental Studies Hybridoma Bank, University of Iowa), anti-col or anti-GFP (Invitrogen). All secondary antibodies were purchased from Invitrogen.

IV. Results

A. Genome wide screen

A screen was performed by crossing flies containing RNAi against known target genes to a fly stock with the MS1096-GAL4 driver and background sensitized by a hypomorphic mutation in *fu* (*fu*^{1}). The *fu*^{1} mutation results in a characteristic wing vein-phenotype: the fusion of the distal portions of wing veins 3 and 4 (Fig. 7B). The progeny resulting from these crosses were scored for changes in the *fu*^{1} phenotype, and classified as strong enhancers, weak enhancers, or suppressors depending upon the nature of the observed effect (Fig. 7). 250 genes were screened as part of this project, which consisted of kinases and phosphatases, proteins that are likely to be involved in the regulation of the Hh signaling pathway due to their enzymatic activity (Graph 1). 20 of these genes resulted in an interesting phenotype when knocked down (Graph 1, Table 1). These 250 genes were part of a larger effort to screen the entire *Drosophila* genome in order to identify novel proteins involved in the Hh signaling pathway.

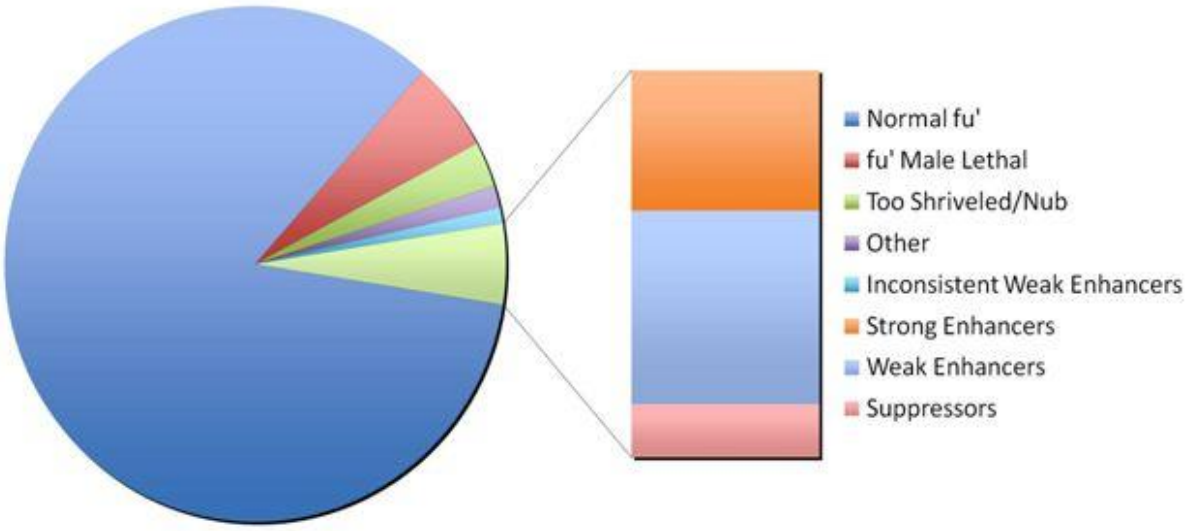


Graph 1. Distribution of phenotypes observed in an RNAi-based screen of the *Drosophila* genome.
The screen was performed in a background sensitized with a hypomorphic mutation in the kinase fused (fu^{1}). Graph displays the results of 250 crosses.

	# Screened	Percentage (%)
Normal fu[1]	173	69.2
Male Lethal	17	6.8
Other	17	6.8
Inconsistent Weak Enhancer	21	8.4
Weak Enhancers	10	4
Strong Enhancers	2	0.8
Inconsistent Suppressors	2	0.8
Suppressors	8	3.2
Total	250	

Table 1. Results from an RNAi-based screen of 250 kinases and phosphatases looking for novel proteins involved in the Hh signaling pathway.

Overall, 11% of the *Drosophila* genome has been screened. Out of 1590 genes, 29 were strong enhancers of the *fu*[1] phenotype and 11 were suppressors (Graph 2, Table 2).



Graph 2. Distribution of phenotypes observed in an RNAi-based screen of the *Drosophila* genome. The screen was performed in a background sensitized with a hypomorphic mutation in the kinase fused (fu^1). Graph displays the results of 1590 crosses, 11% of the genome. Data shown was also collected by Shana Flicker and Ryan Hurtado.

	# Screened	Percentage (%)
Normal fu[1]	1337	84.1
Male Lethal	90	5.7
Too Shriveled/Nub	45	2.8
Other	22	1.4
Inconsistent Weak Enhancer	16	1.0
Strong Enhancers	29	1.8
Weak Enhancers	40	2.5
Suppressors	11	0.7
Total	1590	

Table 2. Results from a genome-wide RNAi-based screen covering 1590 genes. Data shown was also collected by Shana Flicker and Ryan Hurtado.

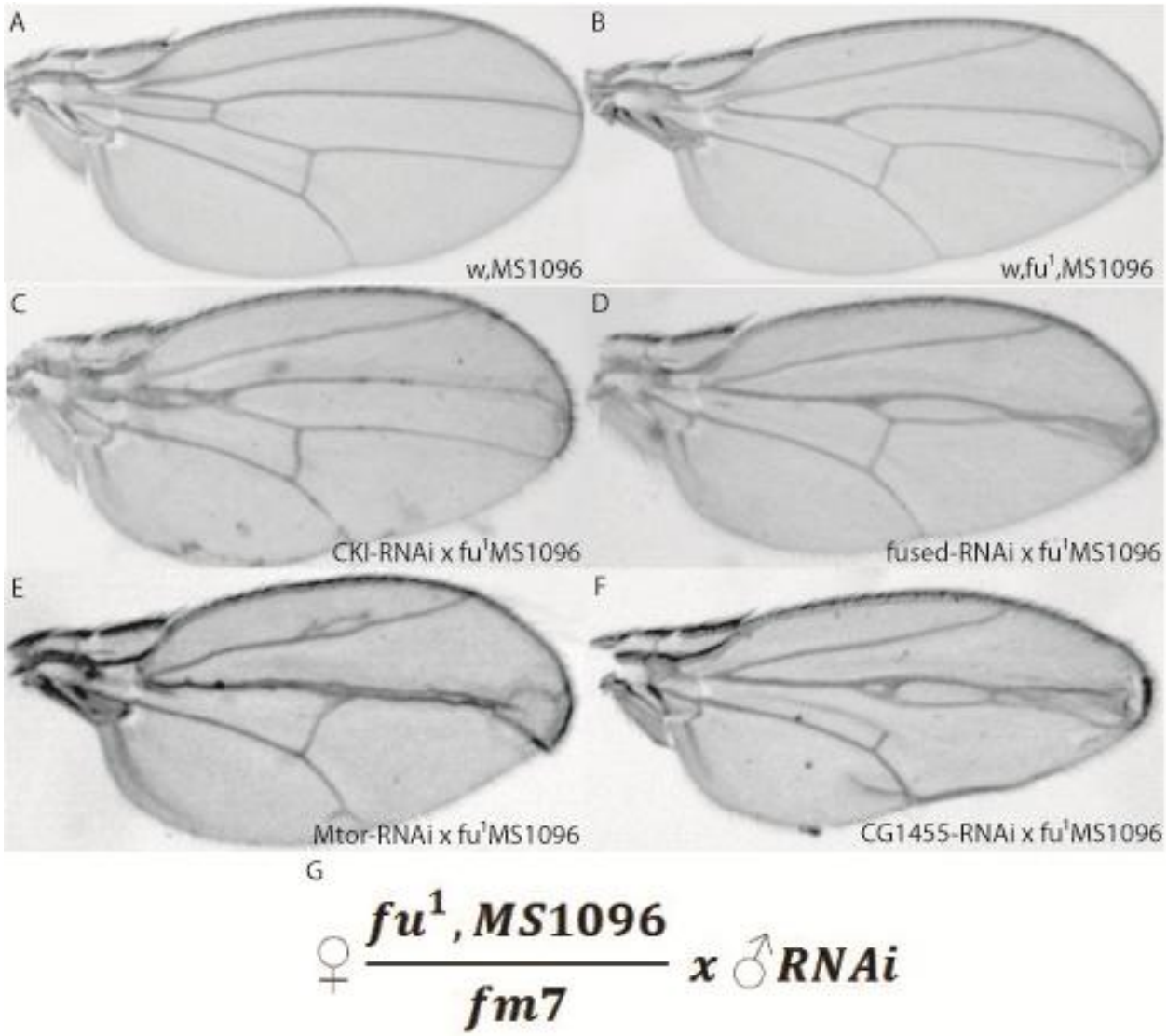


Figure 7. *Drosophila melanogaster* adult wings displaying phenotypic categories observed in the screen. A: Wild-type (WT); B: *fused* MS1096 control (Fu^1); C: Weak Enhancer (WE); D: Strong Enhancer (SE) *megator* (mtor); E: CKI, known Hh pathway member; control; F: Fused, known Hh pathway member control. G: Fly cross.

Having an effect on the fu^1 mutant phenotype suggests that a particular gene may be involved in the Hh signaling pathway. The interesting hits identified by this screen were then analyzed to determine whether their knockdown not only resulted in a change in adult wing phenotype but also if the expression of Hh target genes was altered.

B. Immunohistochemical Assays

The expression of target genes was examined using an immunohistochemical approach in *Drosophila* larval imaginal discs. Levels of the Hh target genes *ptc*, *col*, and *en* were assayed using antibodies specific to their protein products. Flies containing Dpp-LacZ and Ptc-LacZ were used to observe the levels of transcription of *dpp* and *ptc* respectively. RNAi lines were crossed to flies containing Apterous-GAL4, which drove expression of the RNAi in only the dorsal compartment of the wing disc (Fig 9). Changes in gene expression were determined by comparing the dorsal and ventral halves of the disc. Initially, only *dpp*, *ptc* and *ci* expression were analyzed for 40 RNAi lines that were found to have an effect on the fu^1 phenotype in the genome wide screen. Images of all imaginal discs screened can be found in Appendix 2. The gene *megator* (mtor) showed the most interesting results.

$$♀yw;\frac{DppZ, AP-GAL4}{CyO, y+}\ x\ ♂RNAi$$

$$♀w\frac{DppZ, AP-GAL4}{CyO};\frac{UAS-GFP}{Tb}\ x\ ♂RNAi$$

$$♀yw\frac{AP-GAL4}{CyO, y+};\frac{PtcZ}{Tb}\ x\ ♂RNAi$$

Figure 8. Fly crosses performed in order to observe effects of RNAi knockdown on the expression of Hh target genes. Crosses are further described in materials and methods section.

C. Megator

RNAi based analysis. Knockdown of *mtor* resulted in a decrease in Hh target gene expression. Antibody stains for the target genes *dpp* (Fig 9A), *col* (Fig 9B), and *ptc* (Fig 9C) were performed. No effect was observed on the express of Ptc protein levels, however a decrease was seen in *ptc* transcription by using a LacZ reporter (Fig 9D).

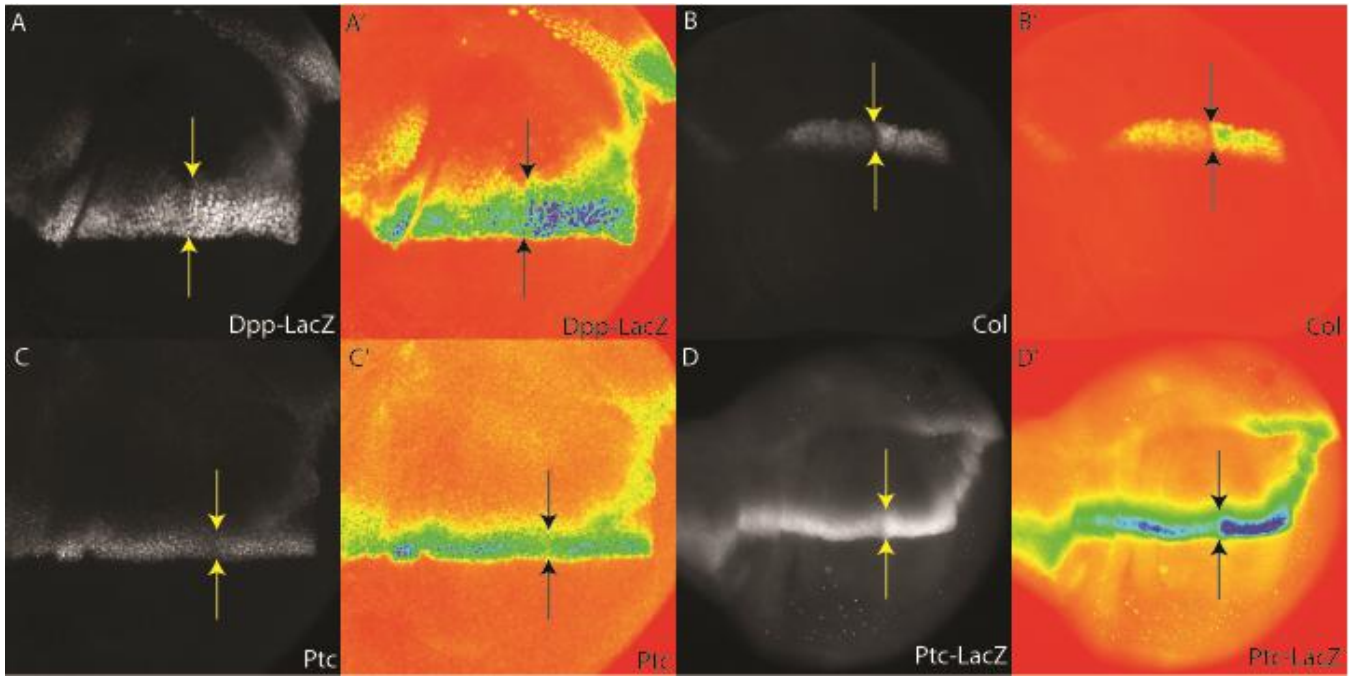


Figure 9. Effects of RNAi-based knockdown of *mtor* on expression of Hh target genes.
RNAi against *mtor* was expressed in the dorsal portion of *Drosophila* imaginal discs using the Apterous-Gal4 driver. The expression of (A) Dpp-LacZ, (B) *collier*, (C) *ptc*, and (D) Ptc-LacZ was visualized with antibody staining. In all images of imaginal discs, dorsal is to the left anterior is up, and arrows mark the boundary between the dorsal and ventral compartments.

The decreases observed in Hh target gene expression could be explained if Mtor was necessary for transcription in general. To test this hypothesis, antibody stains for En were performed. *en* is differentially regulated in the two compartments of the larval wind disc. In the anterior compartment, *en* expression requires Hh signaling, whereas in the posterior compartment expression of *en* is independent of the Hh signaling pathway. Knockdown of *mtor* resulted in a decrease in the expression anterior En. Posterior levels of En were unaffected (Fig 10A). As a second test for whether Mtor was involved in general transcription, Mtor-RNAi was crossed to

flies that contained ubiquitously expressed GFP; no difference was seen between dorsal and ventral compartments (data not shown).

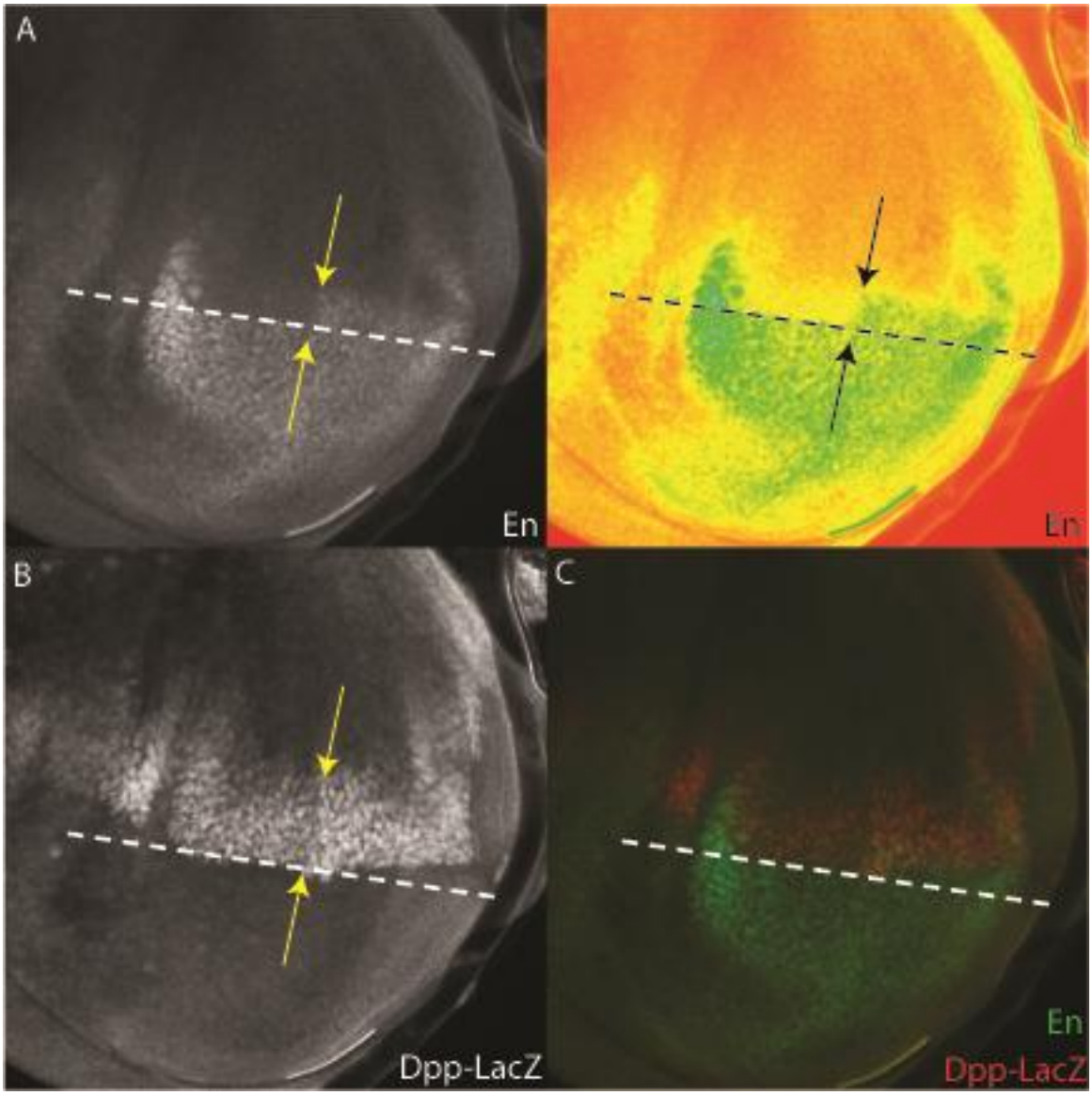


Figure 10. Effects of knocking down *mtor* are specific to targets of the Hh signaling pathway. Antibodies stains for (A) *en*, (B) Dpp-LacZ, and (C) merged. The dashed line represents the boundary between the anterior and posterior compartments.

Since loss of Mtor resulted in a decrease in Hh target genes, a possible explanation is that the transcription factor Ci is affected by Mtor knockdown. Antibody stains for Ci were performed and showed a decrease in protein levels when *mtor* was knocked down (Fig 11A). Previous research has shown that Mtor is involved in the nuclear pore complex (Zimowska et al., 1997).

To test whether loss of Mtor might be affecting Ci's ability to enter the nucleus, wing discs were treated with Leptomycin B (LMB) prior to staining for Ci, which blocks the export of Ci from the nucleus. Even when Mtor was knocked down, Ci was still present in the nucleus (Fig. 11B).

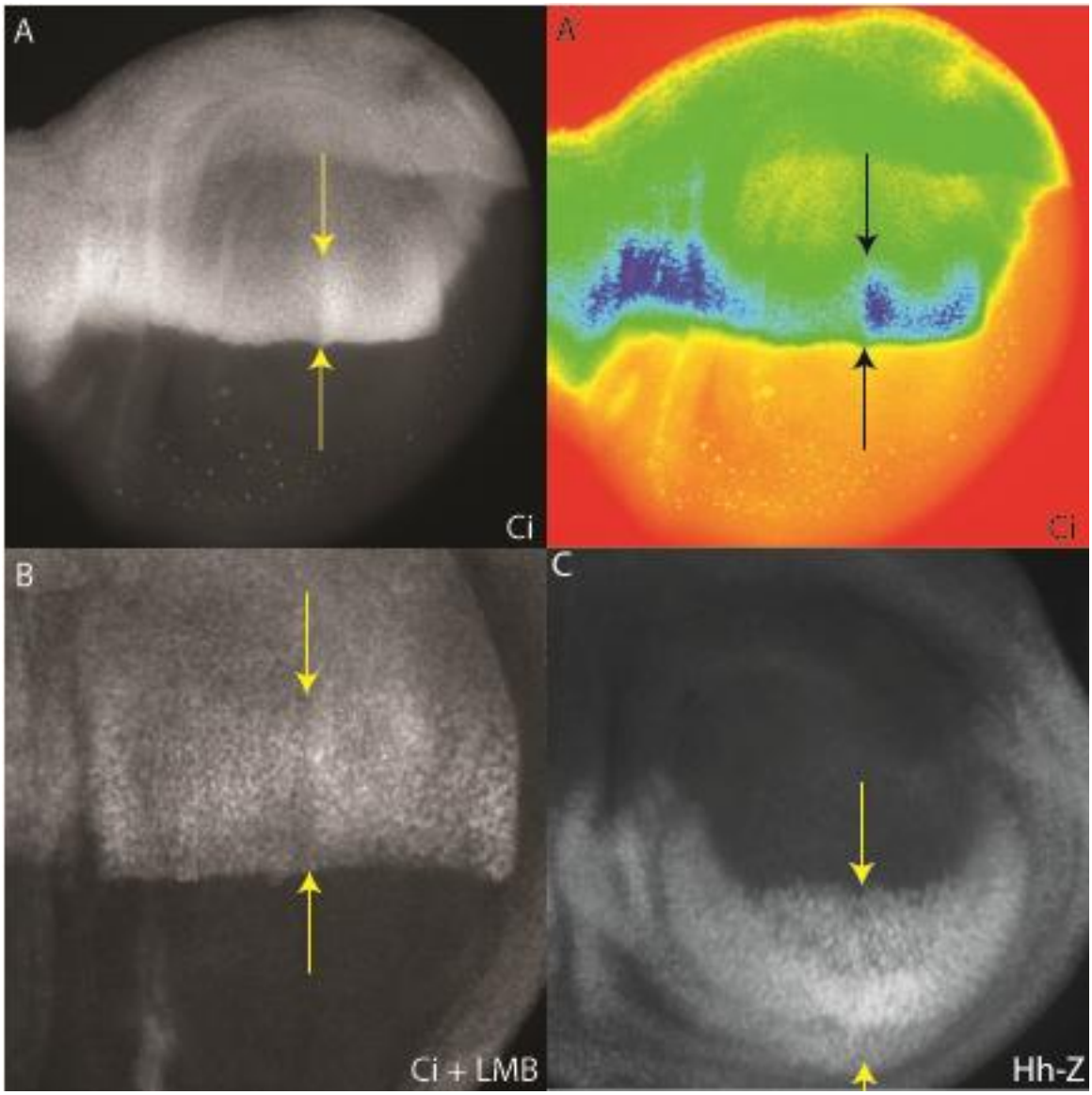
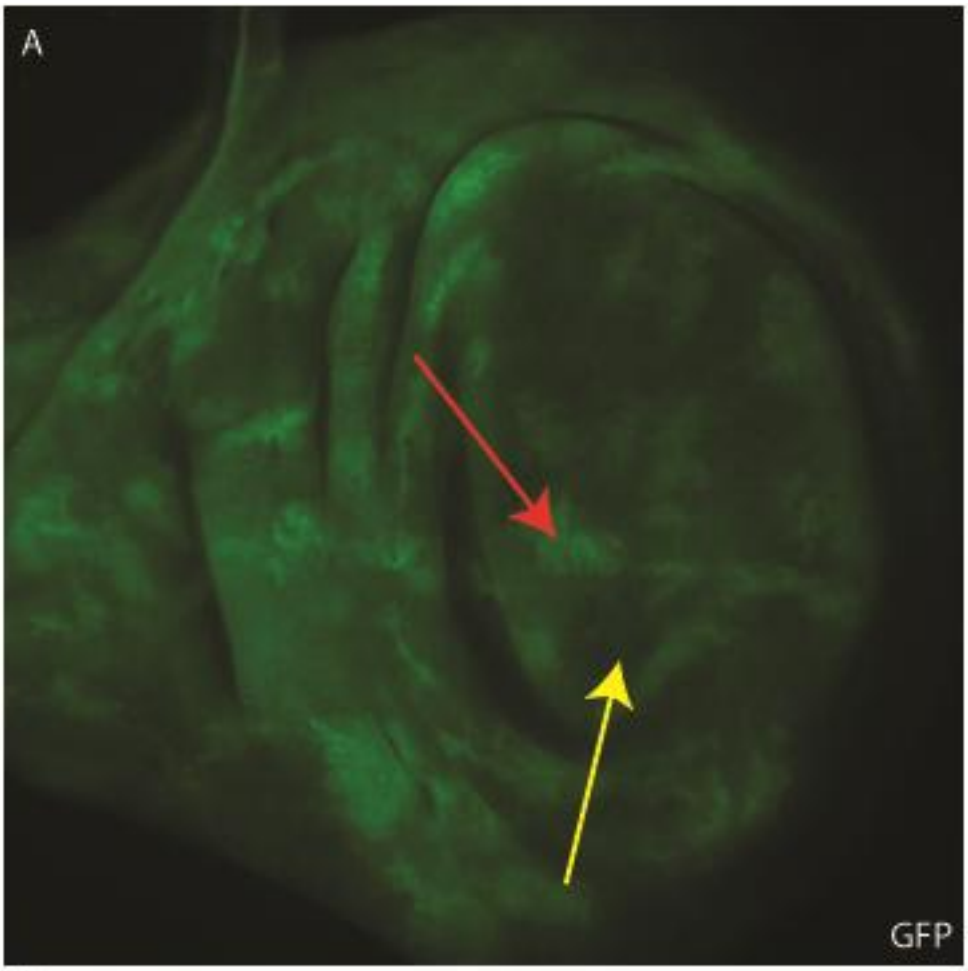


Figure 11. Antibody stains begin to elucidate Mtor's mechanism of action.
mtor-RNAi was expressed in the dorsal portion of each imaginal disc. A: full length Ci, B: full length Ci in discs first treated with LMB, C: Hh-LacZ.

Next, Mtor-RNAi was crossed to flies containing Hh-LacZ in order to begin placing Mtor in the Hh signaling pathway. No noticeable change in the transcriptional levels of Hh was observed when Mtor was knocked down (Fig. 11C).

Analysis of mutations in the endogenous gene. Flies containing a bona fide mutation in *mtor* ($mtor^{k03905}$) were obtained (Spradling et al, 1999). This mutation is homozygous lethal, and so



B

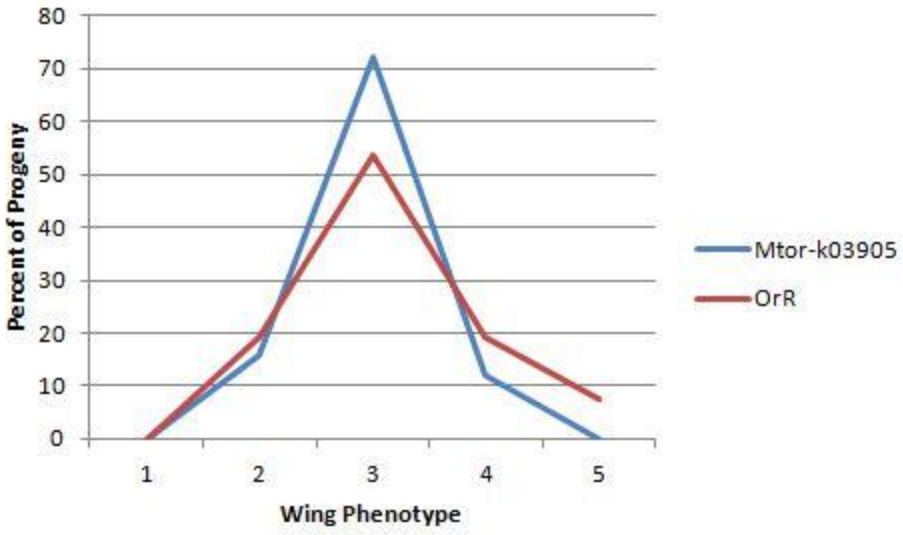
$$♀ywHSFLP; \frac{FRT42D, ubiGFP}{CyO} \; x \; ♂ywHSFLP; \frac{pMtor}{CyO, y+}$$

Figure 12. Attempted clonal analysis using *mtor* real mutant. A: Bright green patches are wild-type cells, darker green are heterozygous for a real mutation in *mtor*, and homozygous mutant cells would be marked by an absence of GFP. Red arrow: wild-type cells, Yellow arrow: heterozygous cells. B: The fly cross performed to make clones.

the FLP-recombinase system was used to induce mitotic recombination to create patches of cells that are homozygous mutant for mtor^{k03905} within the wing disc. Mutant clones were marked by an absence of GFP, and while patches of wild-type (red arrow) and heterozygous mutant cells (yellow arrow) were observed, no homozygous patches of cells were present (Fig 12). In another attempt to show that a bona fide *mtor* mutation interacts with the Hh signaling pathway, Mtor^{k03905} was also crossed to flies containing a *hh* mutation called moonrat (hh^{mrt}), which causes an overexpression of Hh. The *hh^{mrt}* mutation results in an expansion in the anterior portion of the wing, and can range from a slight expansion between veins 3 and 4 to a complete wing duplication. Since the phenotype is variable between individuals, *hh^{mrt}* wings are classified as I through V, depending on severity. The wings of the mtor^{k03905}/+; hh^{mrt}/+ adult progeny from this cross were scored based on vein phenotype and compared to a control cross of *hh^{mrt}* to wild-type flies (Table 3). No statistical difference was found between the two sets of wings (t = 1) (Graph 3). See Appendix 3 for images of scored wings.

	Hh^{mrt} x Mtor^{mut}		Hh^{mrt} x OrR	
Class	# Scored	% of Total	# Scored	% of Total
I	0	0	0	0
II	4	16	5	19
III	18	72	14	54
IV	3	12	5	19
V	0	0	2	8
Total	25		26	

Table 3. Scoring of wing phenotypes from crosses to *hh^{mrt}*.
Wings were scored according to the methods used in Casso et al. The number of wings counted under each phenotypic classification was then normalized to the total number of flies from each cross.



Graph 3. Distribution of wing phenotypes of hh^{mrt} $mtor^{k03905}$ double mutants compared to control. Hh^{mrt} was crossed with either $Mtor^{k03905}$ or OrR. The wings of the progeny were scored according to the system defined by Casso et al, and the distribution of phenotypes is shown. A t test showed no significant difference between the two populations of wings.

V. Discussion

The purpose of this project was to identify novel proteins involved in the regulation of the Hh signaling pathway. By using *Drosophila,* it was possible to perform a genome-wide screen to look for potential candidates. The screen identified a subset of genes that were more likely to be involved in the Hh signaling pathway, which were then subjected to further analysis.

A. The Screen

The screen was performed in a sensitized background using the hypomorphic mutation fu^1, which causes a partial fusion of veins 3 and 4 in adult fly wings. This partial fusion is due to a decrease in activation of the Hh signaling pathway, and so this sensitization allows for the identification of proteins that either increase or decrease activation of the pathway, called suppressors and enhancers respectively. Having a sensitized background was an essential reason for the success of this screen. Previous work has shown that simply knocking down a known component of the Hh signaling pathway, such as CK1, has no adult wing phenotype. However, when knocked down in a background sensitized with fu^1, knockdown of CK1 results in a noticeable rescue of the fu^1 mutant phenotype. Sensitization is necessary for several reasons, one of which is that the Hh signaling pathway is robust and can compensate for partial loss of one part of the pathway. Another reason is that often the proteins being knocked down in the screen had enzymatic activity, so that even when knocked down, the remaining proteins still functioned properly in the pathway. Also, RNAi does not completely abolish gene expression, but instead only leads to a reduction which might not be enough to result in a noticeable phenotypic change. Introducing a sensitizing mutation helps to address these problems. Despite its challenges, RNAi is a valuable tool for performing large screens. It avoids some of the problems found with other methods of decreasing gene expression. Forward genetic techniques such as random mutagenesis

by EMS require genetic mapping in order to identify what gene has been disrupted, which takes time; and the same applies for P-element mediated gene disruption. Another option is to use deletion libraries, as in the screen performed by Casso et al. (2008), however; these deletions can often span multiple genes which makes interpretation of results difficult or could mask genes of interest if a positive and negative regulator are found within the same deleted region. An RNAi-based screen in a sensitized background is a quick and effective method for identifying novel members of the Hh signaling pathway, and its success is shown by the 80 genes identified as affecting the fu^1 mutant phenotype.

The screen was able to pull out known pathway members, such as CK1 and Fu, which validates the screen's ability to identify proteins involved in the Hh signaling pathway and suggests that the novel proteins may also be involved. Initially, the screen focused on kinases, as the enzymatic activity of these proteins makes them likely regulators of a signaling pathway. Later, the screen was expanded to include other regulatory enzymes, such as phosphatases, acetylases, and deacetylases. In the future, screening proteins involved in ubiquitination, SUMOylation, methylation, and other post-translational modifications is likely to be fruitful. However, the screen should also expand to include more proteins that may not have enzymatic activity. For example, one of the hits from the screen, Mtor, is not an enzyme but instead plays a structural role in the cell. Ideally, with enough time, the screen could encompass the entire *Drosophila* genome. The screen successfully identified both positive and negative regulators of the Hh signaling pathway. 11 genes suppressed the fu^1 mutant phenotype when knocked down (Fig. 7C) and 29 genes resulted in a complete fusion of veins 3 and 4, termed a strong enhancer (Fig. 7E) (Table 2). 22 genes were classified as having the "other" phenotype, which was usually a wing that was shorter along the distal axis and had a pinched-looking proximal portion. This

phenotype was termed “kinked” and was determined to only be found in a subset of RNAi lines that all had the same insertion site for the shRNA transgene. Thus, the kinked phenotype was likely an artifact of the insertion site, and so transgenic flies created with the KK site will no longer be used in the screen.

The use of a RNAi-based screen in a sensitized background is not limited to the Hh signaling pathway, but could be adopted as a method of identifying novel proteins involved in any signaling pathway. By changing the mutation used for sensitization, this screening method could easily be adapted for other pathways. For example, a hypomorphic mutation in Frizzled might be used to sensitize a screen for novel Wnt pathway members.

B. Follow-up

Antibody stains looking at levels of Hh target gene expression for 40 of the interesting hits pulled from the screen were performed. However, most genes of interest did not affect target gene expression when knocked down (Appendix 2). One possible explanation could be because RNAi was used for these experiments. Since RNAi only knocks down a target gene, sufficient amount of the protein may be present for the pathway to function. Another possible explanation is that unlike the screen, the antibody stains were not performed in a sensitized background, and just like in adult wings the pathway may be able to compensate for loss of just one component and so gene expression levels appear unaffected. These stains will be repeated in a fu^{1} mutant background in the future. Or the genes may not actually be involved in the Hh signaling pathway. One of the hits analyzed that did show an effect on gene expression was *mtor*.

C. Megator

Mtor appears to be necessary for full expression of Hh target genes. Knockdown of Mtor using RNAi resulted in a decrease in target gene expression. This effect was observed both in

genes that are targets of high levels of Hh, *ptc*, *col*, and anterior *en*, as well as targets of moderate levels of Hh, such as *dpp* (Fig. 9), which suggests that Mtor is involved in the function of the pathway as a whole. While stains for Ptc protein did not show a noticeable affect when Mtor was knocked down, when transcriptional levels were observed using a LacZ reporter, there was a decrease. The lack of an effect seen in Ptc protein is most likely due to the robustness the Hh signaling pathway – the system was able to compensate and still produce a normal amount of protein from fewer transcripts. Another possible explanation for the decreases in target gene expression could be that Mtor is important for transcription in general, in which case loss of Mtor should result in a decrease in the expression of any gene. However, this is unlikely because of the results from antibody stains for En (Fig. 10A). While En in the anterior compartment decreased with knockdown of Mtor, posterior En was unaffected, suggesting that Mtor is not involved in transcription or translation in general. The results from experiments with ubi-GFP also support this finding. All together, these antibody stains suggest that Mtor is necessary for activating the Hh signaling pathway and that its role is Hh specific.

The previous results relied on RNAi for knockdown of Mtor, but there are several concerns with this method. Because of the potential for off target effects, it is not possible to say that Mtor is actually involved in the Hh signaling pathway until more certain results can be obtained. To address these problems, experiments with $Mtor^{k03905}$, a hypomorph of *mtor*, were performed.

First, $Mtor^{k03905}$ was crossed to fu^{1},MS1096, however because $Mtor^{k03905}$ is homozygous lethal only progeny heterozygous for $Mtor^{k03905}$ could be obtained. No effect was observed on the fu^{1} mutant phenotype, most likely because a partial loss of Mtor was not sufficient to compromise the Hh signaling pathway and the flies were able to compensate. In order to

circumvent the problem of lethality, Mtor^{k03905} was used in an attempt to make clones via mitotic recombination (Fig. 12). Imaginal discs containing clones were also stained for Ci in an attempt to see an effect in the cells that are homozygous for the mutation in *mtor*. While it was apparent that mitotic recombination did occur, no patches of homozygous mutant cells were observed. Previous work on Mtor has shown that cells homozygous for Mtor^{k03905} have noticeable growth defects; they are smaller and slower to undergo mitosis (Qi et al., 2004). The lack of homozygous mutant clones is most likely due to the fact that these cells were being outcompeted by their neighbors, which were either wild-type or only heterozygous for Mtor^{k03905}. One possible way to get around this problem is to use flies with a mutation called Minute, which compromises ribosomal function and causes cells to grow more slowly. After mitotic recombination, all the cells that do not receive two copies of Mtor^{k03905} will contain Minute, which will hopefully allow the homozygous Mtor^{k03905} clones to grow large enough to be observed. These experiments with Minute are currently being performed, and hopefully a decrease in Ci occurs with homozygous mutant clones, showing that a loss of Mtor is actually responsible for the observed effect.

Another experiment which attempted to show that a bona fide mutation in *mtor* interacts with the Hh signaling pathway was to cross Mtor^{k03905} to flies containing the *hh*^{mrt} mutation. The hope was that because Mtor^{k03905} is a hypomorph it would rescue the hypermorphic *hh*^{mrt}. The progeny resulting from this cross did not show any significant difference in phenotypic distribution than the progeny from a control cross of *hh*^{mrt} to OrR, a wild-type fly stock. Most likely the lack of an interesting result was again due to the fact that the progeny were only heterozygous for Mtor^{k03905}, and because the mutation is only a hypomorph the resulting decrease in the amount of Mtor was insufficient. Theoretically, it would possible to perform a

cross of the f_1 in order to produce flies that are homozygous for Mtor^{k03905}, and since they would also contain *hh*^{mrt}, the homozygotes might survive. However, so far attempts to do this have been unsuccessful.

The best way to determine whether Mtor is part of the Hh signaling pathway is to elucidate the mechanism by which it acts. Some information is already known about *mtor*: it encodes a 260-kDa protein that contains a large coiled-coil domain. Previous work has shown that Mtor associates with the proteins Skeletor and Chromator as part of the mitotic spindle (Qi et al., 2004). Stains performed in cell culture with an antibody specific for Mtor have shown that it also localizes to the nuclear envelope during interphase, where it is thought to be part of the nuclear pore complex (Zimowska et al., 1997). More experiments should be done to look at the potential involvement Skeletor and Chromator, since they associate with Mtor they may also be involved in the Hh signaling pathway. First, RNAi against these two proteins should be crossed into the screen. If either of them affects the *fu*^{1}mutant phenotype, then antibody stains should be performed to see if they affect target gene expression as well.

In order to begin to elucidate Mtor's role in the Hh signaling pathway, more antibody stains were performed using RNAi to knock down Mtor in imaginal discs. First, the transcription of Hh was looked at by using a LacZ reporter. This experiment was performed in order to begin placing Mtor within the cascade of the Hh signaling pathway; if knock down of Mtor resulted in a decrease in Hh expression, then that would mean Mtor most likely played a role in the cells of the posterior compartment where Hh is produced. Knockdown of Mtor did not have an obvious effect on Hh-LacZ (Fig. 11C). This result also supports the conclusion that Mtor is not involved in general transcription. Since only the levels of Hh transcript were looked at in theory it could

be possible that Mtor is regulating Hh post-transcriptionally; it is more likely that Mtor is functioning downstream of Hh in the pathway.

Next, an antibody stain for Ci was performed, which showed a decrease in Ci when Mtor was knocked down. As a transcription factor, Ci is the means by which the Hh signaling pathway turns on target gene expression. In order to control when and where genes are expressed, Ci is regulated in many different ways. First, Ci is regulated at the level of its own transcription and translation. Once translated, Ci can either remain in its full-length form or be processed by the proteasome into a truncated repressor. What features of Ci determine whether it is targeted to the proteasome was another project worked on by the author and is discussed in Appendix 3. Finally, the access of Ci to its target DNA is regulated at the level of nuclear import. As previously mentioned, full-length Ci contains both nuclear import and export signals. Since Mtor has been shown to be part of the nuclear pore, and this complex of proteins is responsible for regulating traffic into and out of the nucleus, a likely hypothesis is that Mtor is involved in helping Ci to enter the nucleus (Fig. 13). This hypothesis would explain the decreases observed in Hh target gene expression, since without Mtor, Ci would be stuck in the cytoplasm, unable to turn on any of its target genes. In order to test this hypothesis, imaginal discs were treated with LMB and then stained for Ci. Normally, the export signal is more robust than the signal for import, and so at equilibrium Ci is observed in the cytoplasm and appears absent from the nucleus. LMB blocks nuclear export, and so Ci remains in the nucleus. If Mtor is necessary for Ci to enter the nucleus, knocking down Mtor should result in cytoplasmic Ci, even when discs are treated with LMB. No change in the nuclear localization of Ci was observed with LMB treatment and knockdown of Mtor (Fig. 11B), however this result does not necessarily mean the hypothesis is incorrect. Since RNAi was used Mtor was only knocked down and so import of Ci might have been slowed, but

not completely stopped, which would not have been noticeable after a longer amount of time. Or Mtor might act in another manner.

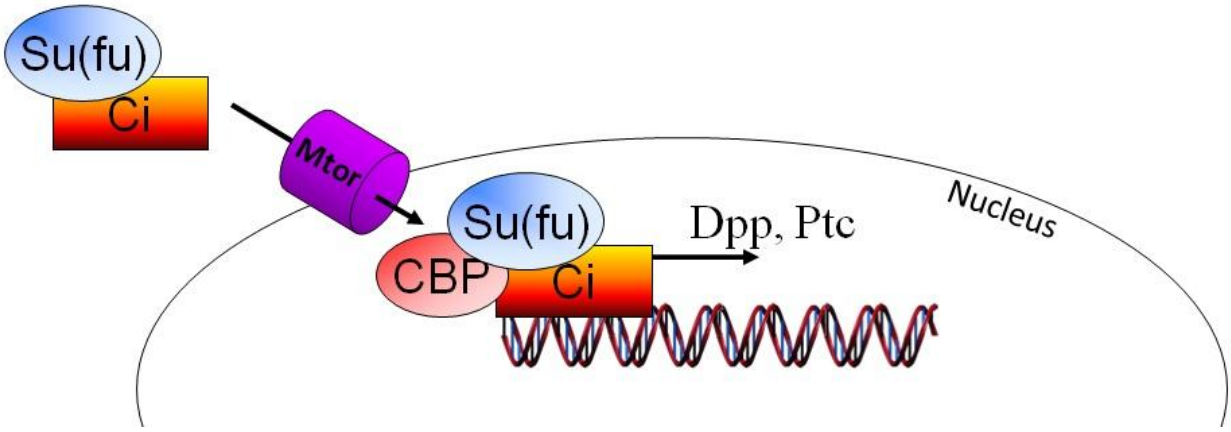


Figure 13. Hypothetical role of Mtor in the Hh signaling pathway.

Based on previous work by Zimowska et al. (1997) and Qi et al. (2004) showing that Mtor is located at the nuclear periphery during interphase and that it is known to associate with the proteins of the nuclear pore, Mtor could be involved in regulating the import of Ci into the nucleus, thus affecting the transcription of target genes.

Even in the LMB treated wing discs, an overall decrease in Ci is observed just like in untreated wing discs. Mtor could be having an effect on the amount of Ci present which would also affect the levels of target gene expression. Currently, an experiment to look at the effect of knocking down Mtor on the transcriptional levels of Ci is being performed by using a LacZ reporter coupled to *ci*. It is possible that Ci-LacZ will show a more robust effect than just staining for Ci protein, as was the case with Ptc and Ptc-LacZ.

VI. Acknowledgements

First, I would like to thank Professor Holmgren for all of his guidance. As a freshman, I decided to e-mail my roommate's Biology professor in the hopes of getting research experience. Now almost four years later, I look back and realize that I got so much more than just another item for my CV. I have learned so much since my first summer of research and that would not have happened without your continued support.

I have to thank Ryan Hurtado for being my graduate student, mentor, and friend. Without you constantly pushing me I know I would not be where I am today. You not only showed me how to do research, you made sure I took advantage of every opportunity to become a better scientist – coercing me to go to talks and even to give one myself. Best of all you made lab something to look forward to every day, even if it was the weekend. Thank you for helping me with experiments, reading through all of my writing (even the attempts I made the first year that were absolutely terrible), going over posters, preparing me for graduate school, and telling me that everything is going to be fine. If I could say thank you enough times this thesis would probably be about ten pages longer.

Also, I would like to thank the other members of the lab, past and present who helped with the genome-wide screen and follow-up experiments: Erin Kennedy Schrader and Shana Flicker.

I would like to acknowledge the funding I have received from the Department of Biological Sciences, Weinberg College of Arts and Sciences, and Northwestern Summer Research Grant.

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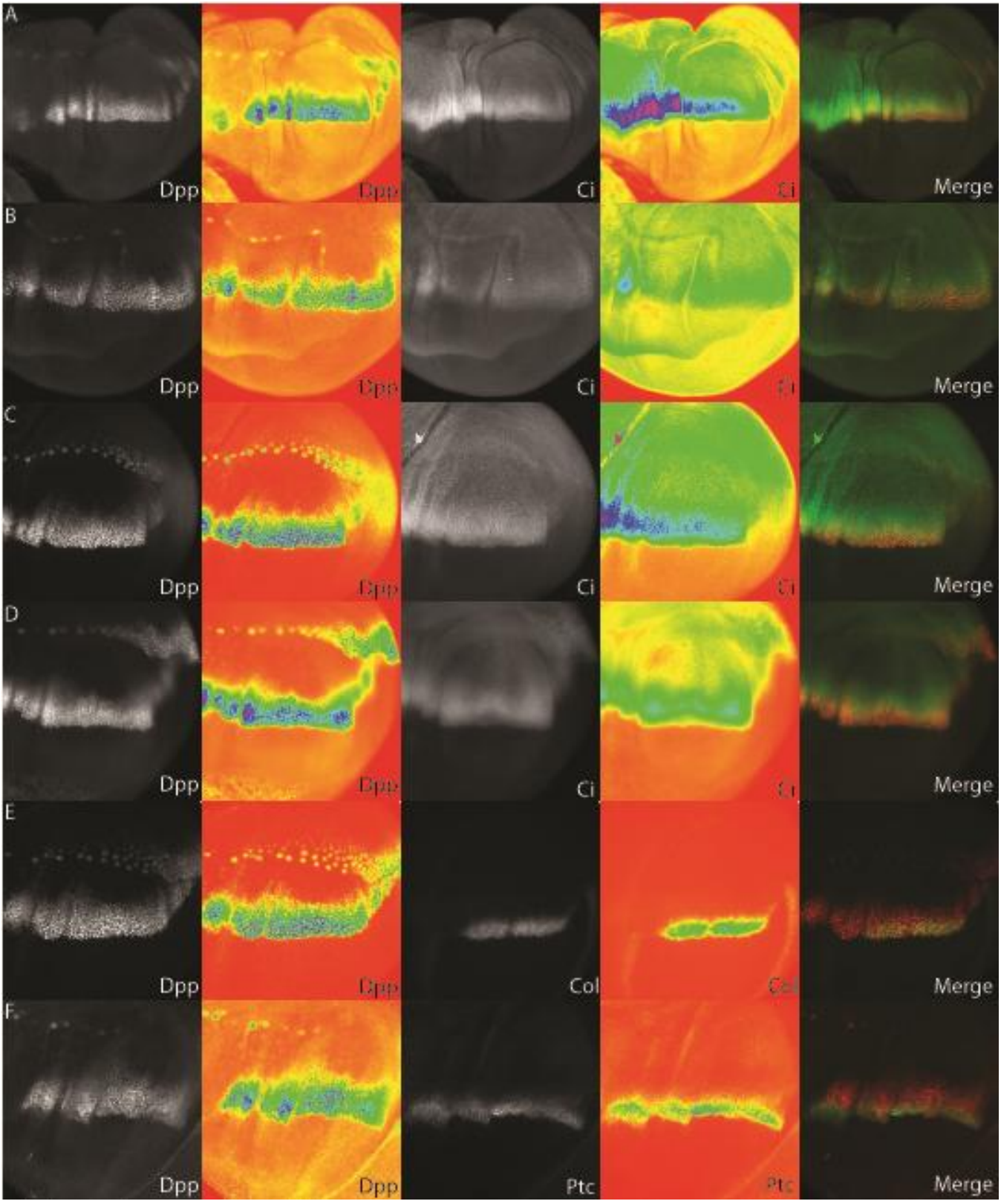
Appendix 1

Immunohistochemical Data for Interesting Hits from Genome-wide Screen

Flies containing UAS-Mtor RNAi were crossed to an Apterous-GAL4 line to target knockdown only in the dorsal portion of the wing disc. Larva from the cross were then dissected, antibody-stained, and imaged using fluorescence microscopy. In all images anterior is up and dorsal is to the left.

A. 1155 (Dpp, Ci)
B. 1455 (Dpp, Ci)
C. 1637 (Dpp, Ci)
D. 2859 2 (Dpp, Ci)
E. 2859 3 (Dpp, Col)
F. 3998 (Dpp, Ptc)
G. 4213 (Dpp, Ci)
H. 4914 (Dpp, Ptc)
I. 4965 (Dpp, Ci)
J. 5263 2 (Dpp, Ci)
K. 5263 3 (Dpp, Ptc)
L. 5784 (Dpp, Ci)
M. 6235 (Dpp, Ci)
N. 7111 (Dpp, Ptc)
O. 7134 (Dpp, Ci)
P. 8485 (Dpp, Ci)
Q. 8651 2 (Dpp, Ci)
R. 8651 3 (Dpp, Ptc)
S. 8888 (Dpp, Ci)
T. 10543 1 (Dpp, Ptc)
U. 10543 2 (Dpp, Ci)
V. 10574 (Dpp, Ci)
W. 11971 2 (Dpp, Ci)
X. 11971 3 (Dpp, Ptc)
Y. 4491 (Dpp)
Z. 12512 2 (Dpp)

AA. 12512 1 (Dpp, Ci, Ptc)
BB. 13197 (Dpp, Ci)
CC. 14317 3 (Dpp, Ptc)
DD. 14317 2 (Dpp, Ci)
EE. 15388 1 (Dpp, Ptc)
FF. 15388 2 (Dpp, Ci)
GG. 18214 (Dpp, Ptc)
HH. 18375 2 (Dpp, Ptc)
II. 18375 3 (Ci)
JJ. 17441 (Dpp)


A
Dpp
Dpp
Ci
Ci
Merge
B
Dpp
Dpp
Ci
Ci
Merge
C
Dpp
Dpp
Ci
Ci
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D
Dpp
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Dpp
Ptc
Ptc
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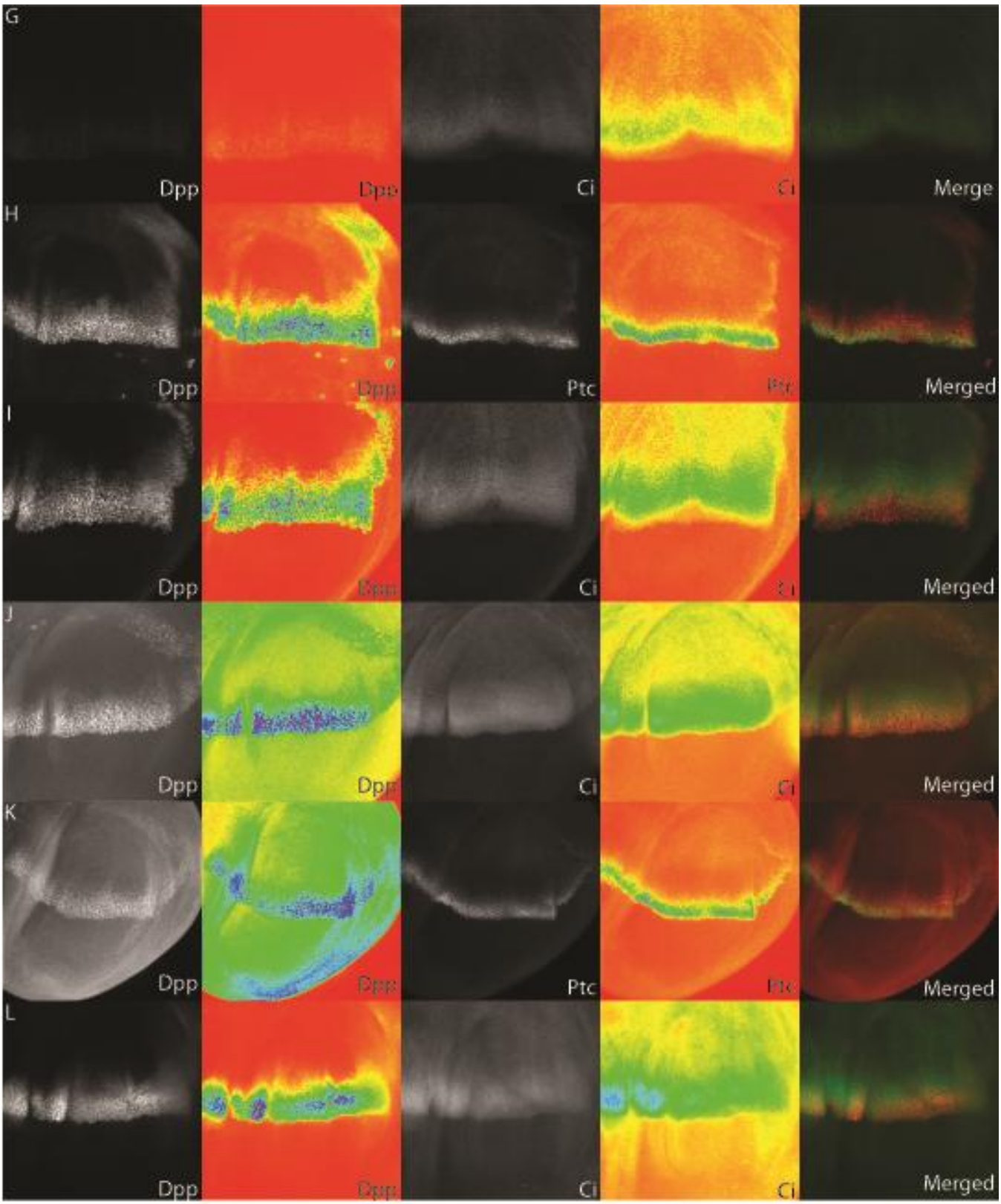
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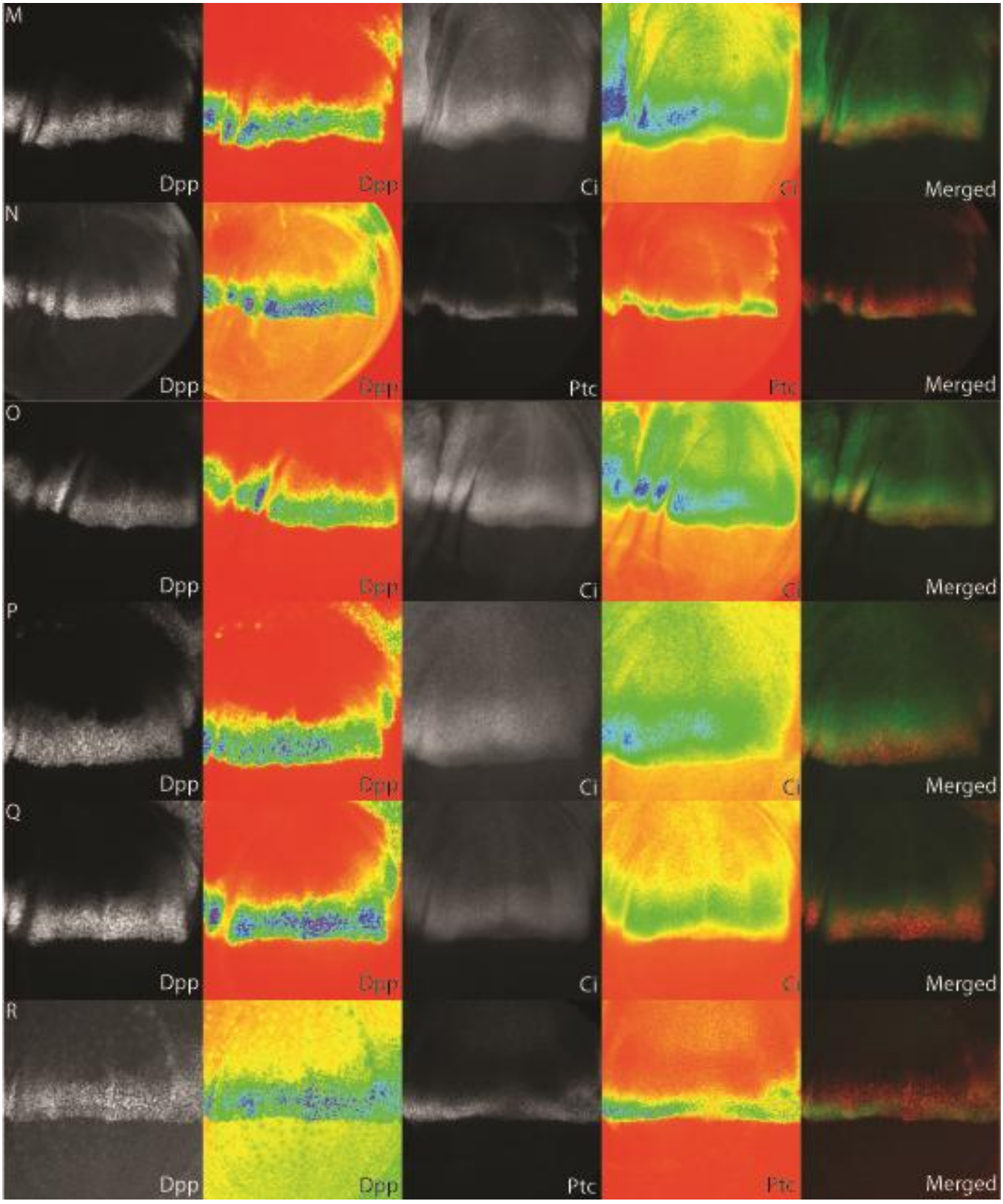
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Merged
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Dpp
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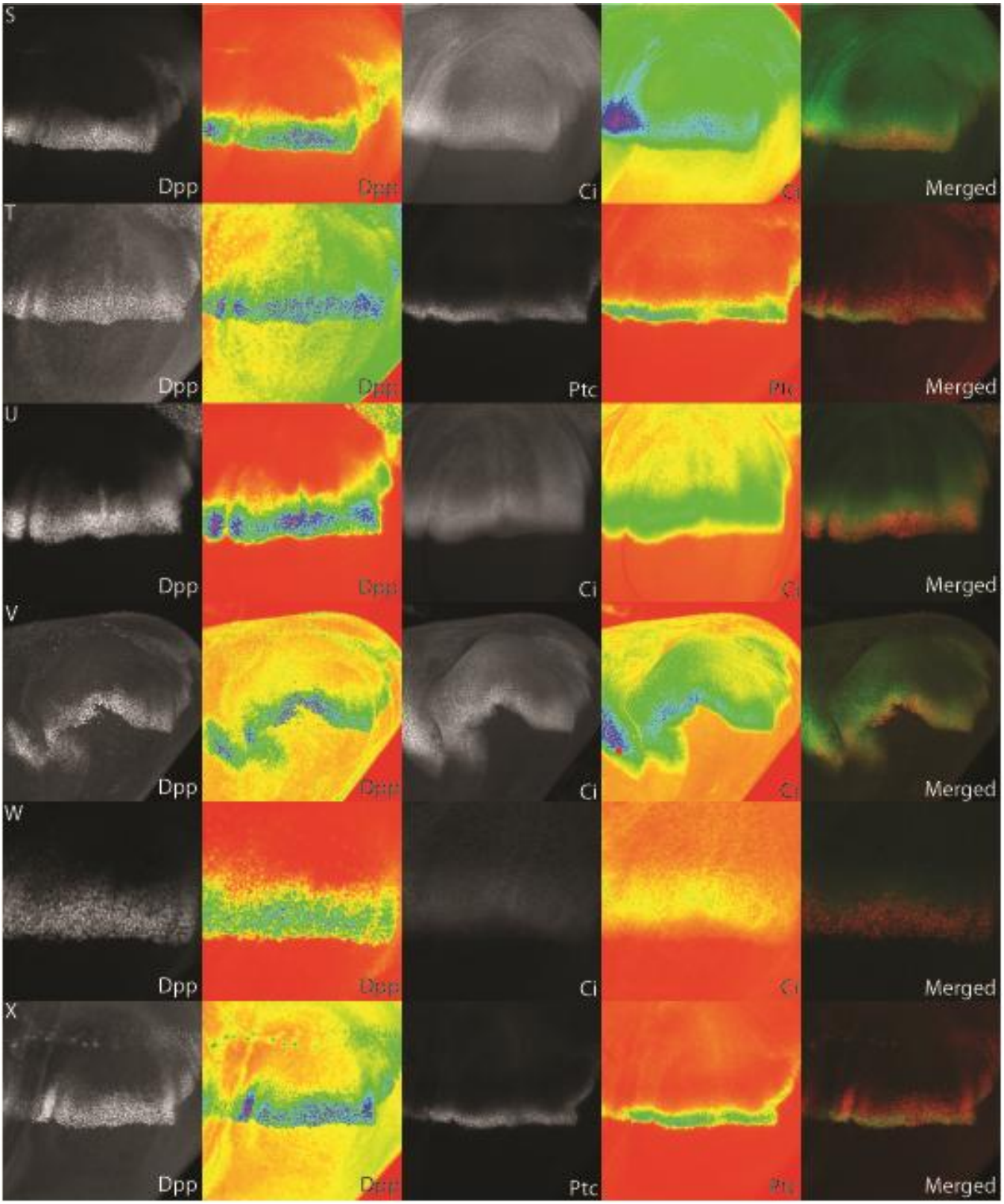
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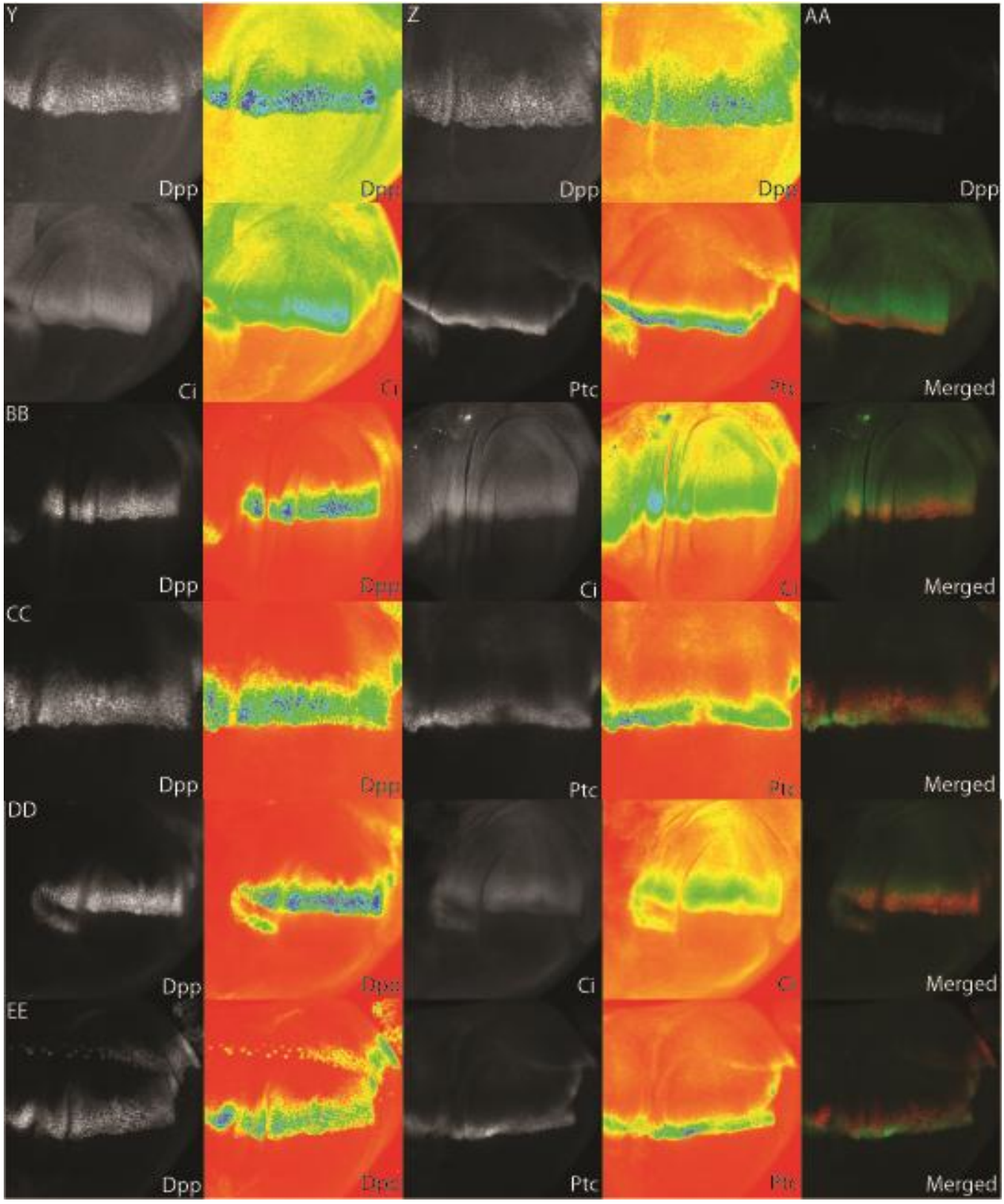
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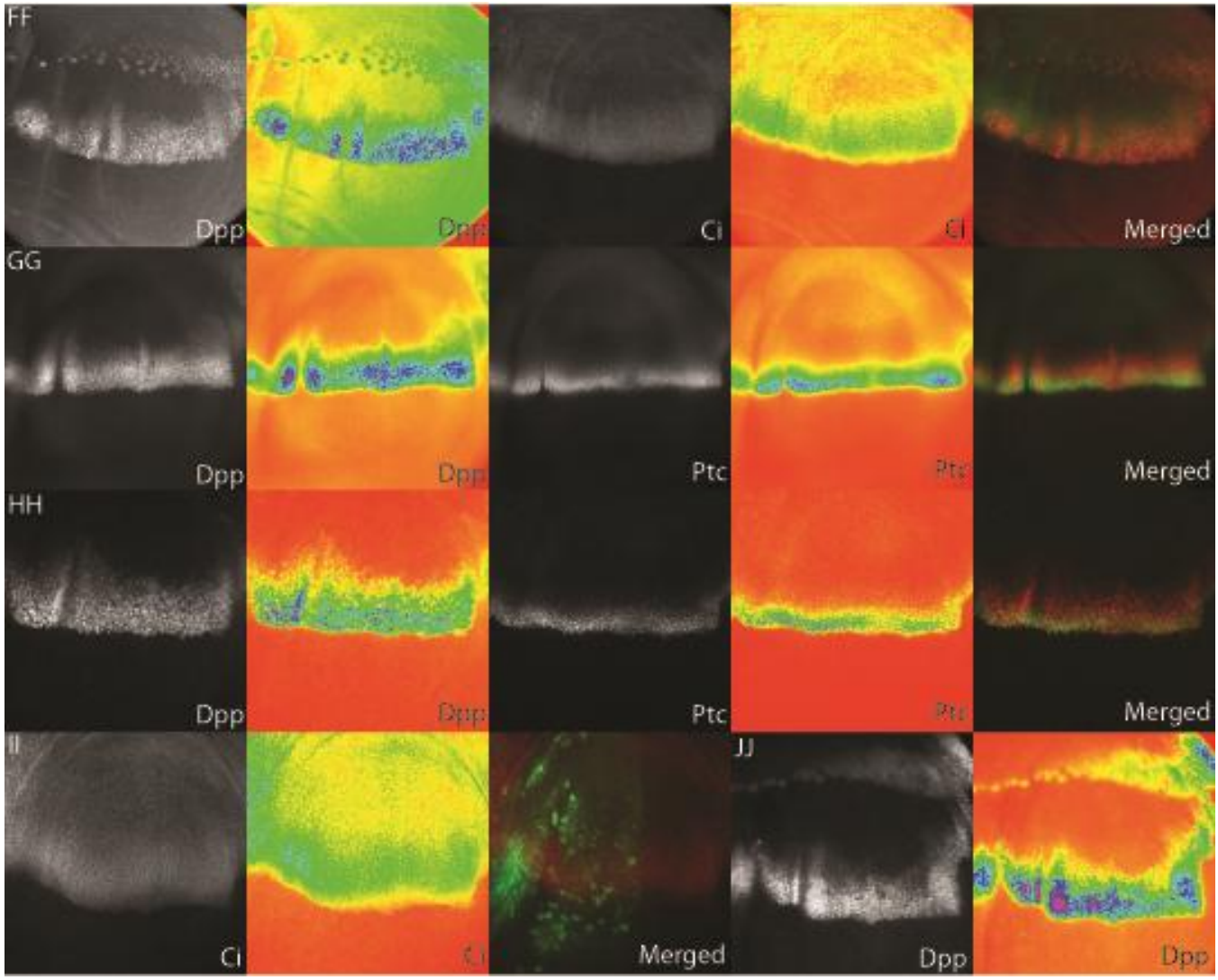
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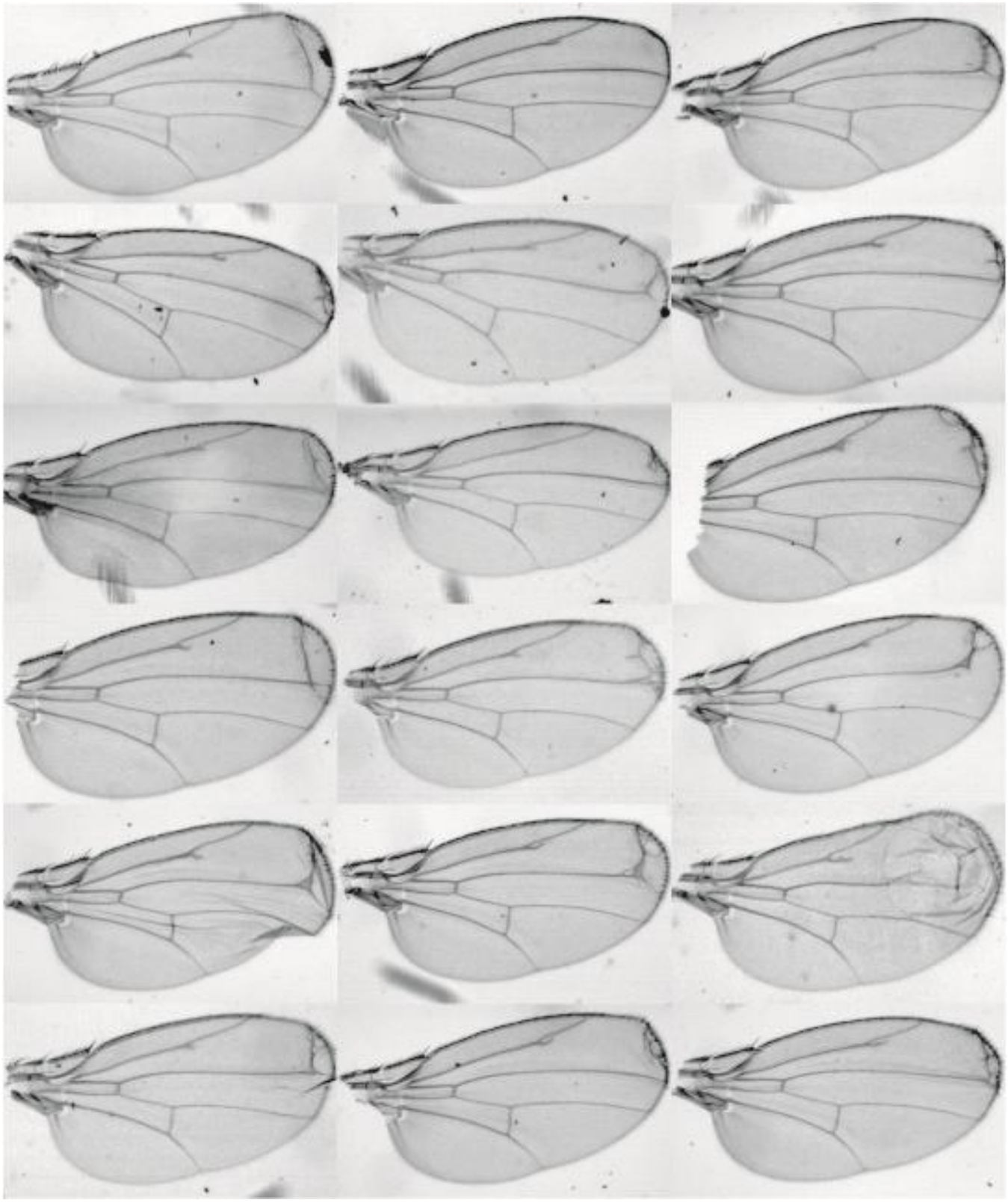
Ptc
Merged
II
Ci
Ci
Merged
JJ
Dpp
Dpp

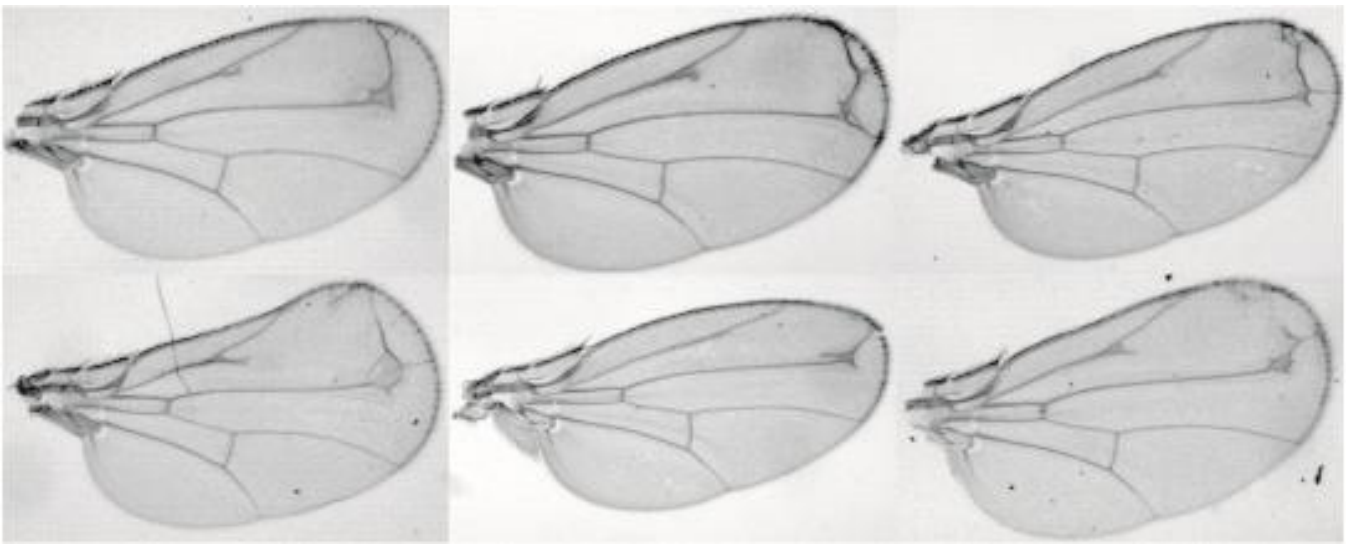
Appendix 2

Data from Hh^{mrt} Experiments

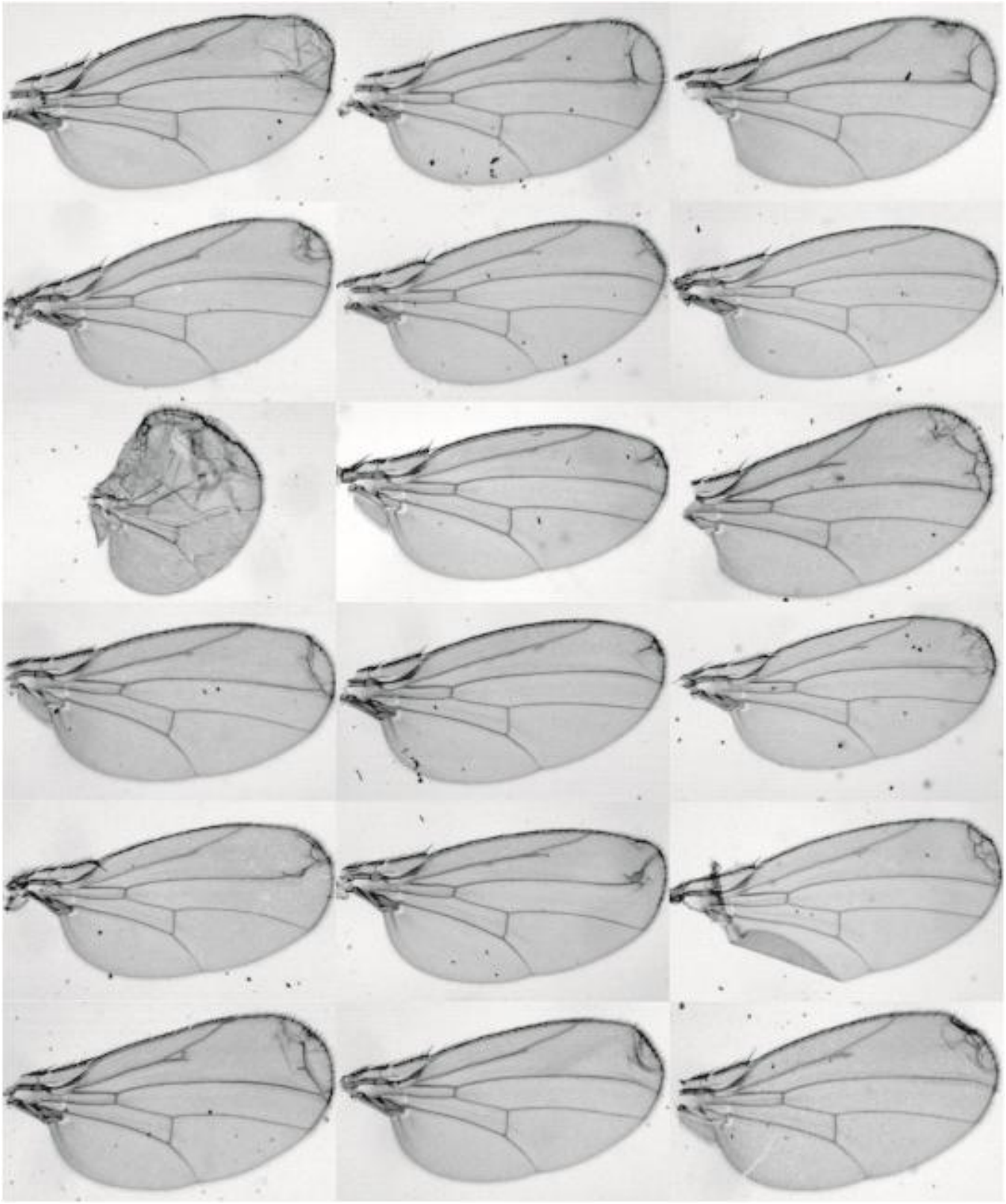
Wings clipped and imaged from progeny of *hh*^{mrt} crossed to either mtor^{k03905} or OrR.

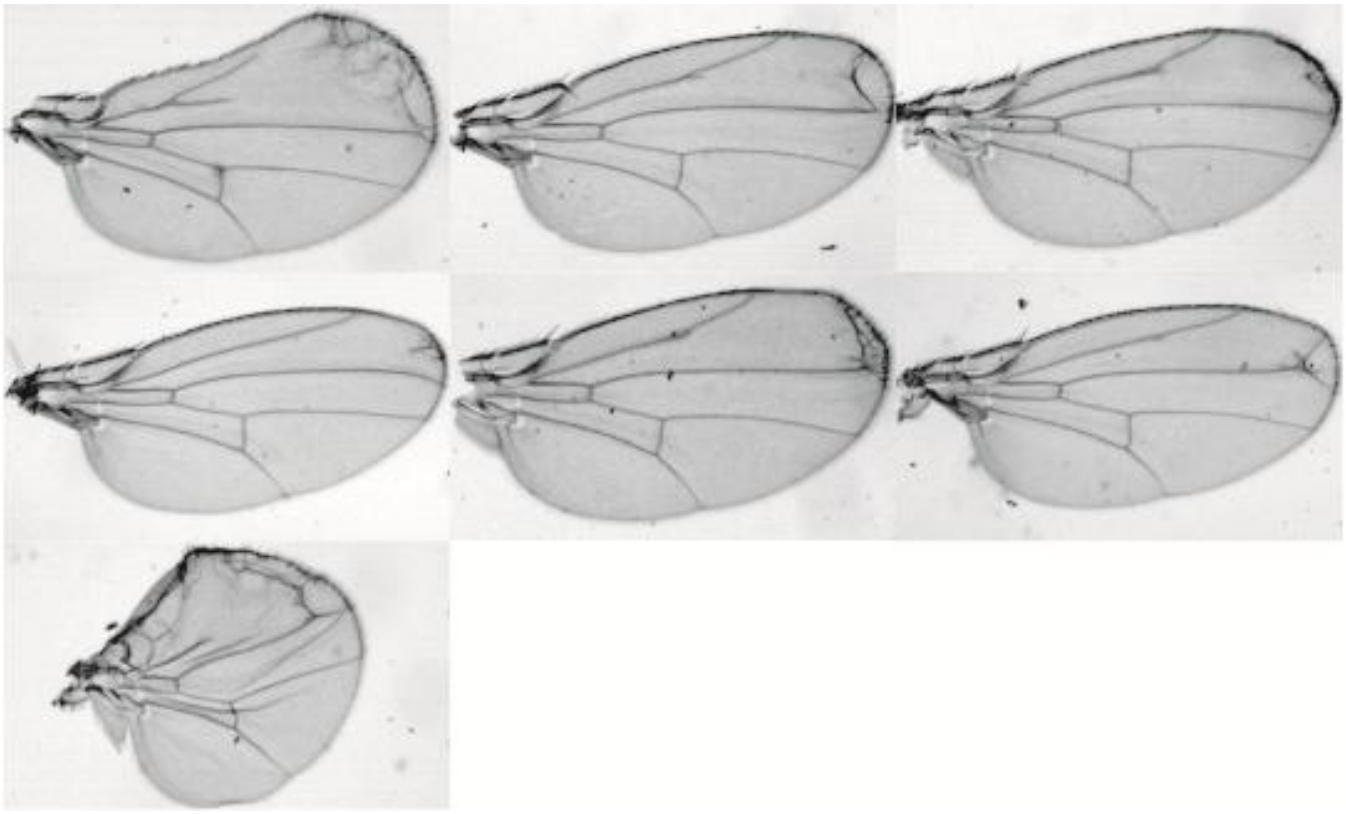
hh^{mrt} x mtor^{k03905}





hh^{mrt} x OrR





Appendix 3

Investigation of the consequences of Gli manipulation *in vitro*

Gli1, 2, and 3 are the mammalian homologues of the protein Cubitus interruptus (Ci) which is found in *Drosophila melanogaster*. In the presence of the Hh ligand, the Hh pathway is on because Ci activates Hh target genes. However, when there is no Hh ligand present, the Hh pathway must be kept off and thus Ci has to repress Hh target genes (Tian et al, 2005). Ci is able to accomplish both contradictory roles because when low levels of Hh are present, Ci undergoes partial degradation or processing by the proteasome, leaving a portion of the protein intact which functions as a repressor of Hh target genes. The proteasome is an organelle responsible for the degradation of intracellular proteins, including transcription factors. In order to be degraded, a protein must first be conjugated to ubiquitin, a small protein that attaches to lysine residues (Albetrs et al, 2004). Then, more molecules of ubiquitin combine to form a chain that binds to the proteasome, which then unfolds the protein using energy from ATP. The proteasome breaks specific peptide bonds in the protein, resulting in a set of smaller peptides that exit the proteasome and can be recycled within the cell (Albetrs et al, 2004). In the case of Ci, the proteasome does not degrade the entire protein; instead it degrades the C-terminus, but then ceases degrading part way through and releases an N-terminal fragment (Wang & Price, 2008). Ci is very unique because it is one of a small group of proteins that are processed by the proteasome, and the precise mechanism of this processing is not well understood.

The function of Ci is conserved in the three Gli proteins; Gli1 and Gli2 are activators of Hh target genes, while Gli3 acts as a repressor. Like its *Drosophila* counterpart, Gli3 is also processed by the proteasome. This project focused on Gli1 and Gli3, specifically what differences exist between the two proteins that cause Gli3, and not Gli1, to be partially degraded.

Previous work with Ci has shown that there are likely two components that cause processing – a low complexity sequence composed of only a few different amino acids followed by a tightly folded domain in the direction of proteasome movement (Tian et al, 2005). The theory is that the proteasome has difficulty "gripping" the simple sequence so when it reaches the folded domain it can no longer effectively "pull" on the polypeptide chain, which is then released. These same two components are also found in Gli3 and the assumption is that they are required for processing.

Seven constructs were built that consisted of various combinations of Gli1 and Gli3 in order to determine which portions of Gli3 are required for processing (Fig. 14A). Three of the constructs were controls; in order to show that exchanging the N termini between Gli1 and Gli3 had no effect on processing (Fig. 14B). This would verify the assumption that the low complexity sequence and tightly folded domain, which are in the C terminus, are the only elements required for processing. The other control construct was a deletion of the region containing the protein kinase A (PKA) phosphorylation sites in Gli3 (Fig. 14C). The PKA sites are ultimately required for ubiquitination, so Gli3 lacking these sites should not be efficiently targeted to the proteasome and thus should not be processed.

The other four constructs were focused on determining what part of Gli3 is required for processing, and which regions are sufficient to induce processing of Gli1, which is not processed in its wild type form. Two constructs were built that swapped the region containing the PKA phosphorylation sites and adjacent lysines between Gli1 and Gli3 (Fig. 14D).

The two remaining constructs involved the Gli3 region of low complexity. Gli3 contains two stretches of simple sequence, one rich in serine residues, and the other in proline. The serine-rich

region had previously been deleted, and so one construct was designed to delete only the proline-rich region, and the last had both stretches of simple sequences removed (Fig. 14E).

Four of the constructs were created by using the InFusion system, which allowed portions of Gli1 to be swapped into Gli3 and vice versa. Primers were designed for the amplification of the Gli N-termini and PKA site/adjacent lysine inserts as well as the linearization of the Gli1 and Gli3 vectors which removed the endogenous portion of the Gli protein that was going to be replaced by the insert. Each vector and insert pair were fused together using the InFusion enzyme, then transformed into and isolated from Stellar competent cells. The Gli3 region deletions were created by site-directed mutagenesis. After being sequenced, the all of the constructs were ligated into pcDNA and transfected into HEK293T cells where proteasomal processing was induced. Presence of a partially degraded Gli3 protein was then assayed by western blot; so far this has been completed for one construct.

The seven constructs were completed by the end of this project. The deletion of the phosphorylation sites in Gli3 and the insertion of the N-terminus from Gli3 into Gli1 could not be created by the previously described methods. Results were obtained from the processing of one of the constructs in cells (Fig. 14F). When both stretches of simple sequence were deleted from Gli3, processing by the proteasome was greatly reduced, which suggests that the region of low complexity is required for partial degradation.

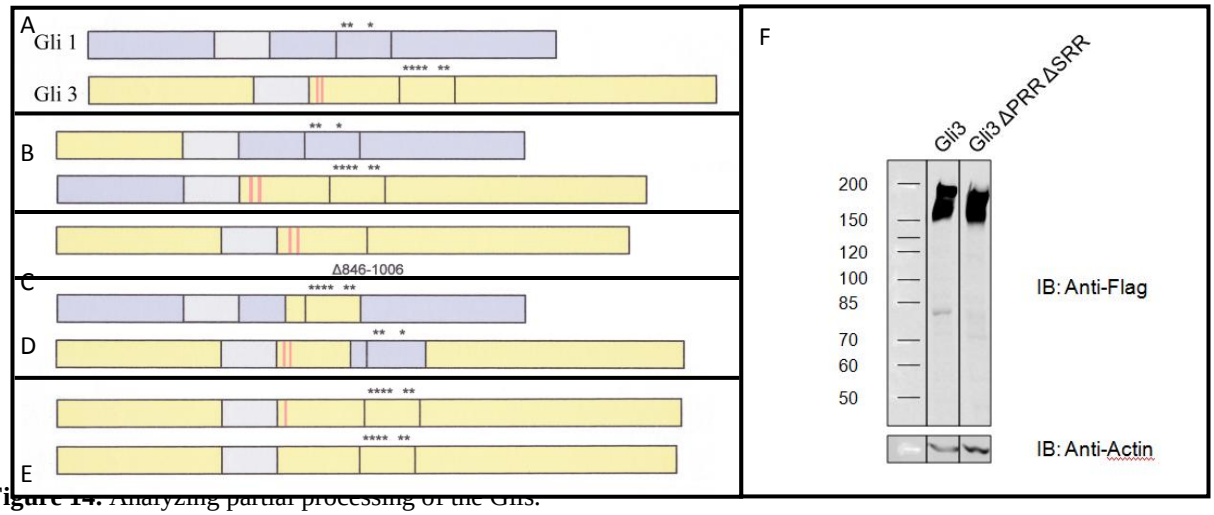


Figure 14: Analyzing partial processing of the Glis.

A: Wild type Gli1 (blue) and Gli3 (yellow) proteins. The gray region represents the tightly folded domain containing the five zinc fingers, followed by the stretches of simple sequences (pink), and then the region containing the PKA phosphorylation sites (stars above the protein).

B: Gli3 construct in which the PKA sites (amino acids 846-1006) are deleted.

C: Swaps of the N-termini between Gli 1 and Gli3. Top: Gli1 with Gli3 1-479 inserted. Bottom: Gli3 with Gli1 1-234 inserted.

D: Swaps of the region containing the PKA phosphorylation sites and adjacent lysines between Gli1 and Gli3. Top: Gli3 with Gli1 468-701 inserted. Bottom: Gli1 with Gli3 846-1006 inserted.

E: Western blot of wild type Gli3 and Gli3 with both simple sequences deleted (Gli3 ΔPRR ΔSRR). Wild type Gli3 is processed by the proteasome into a smaller fragment of about 83 kDa. Gli3 ΔPRR ΔSRR is not efficiently processed by the proteasome and very little fragment is formed.