A Genetic Screen in *Drosophila* to Identity Novel Components of the

Hedgehog Signaling Pathway

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Abstract

The Hedgehog signaling pathway plays an essential role in the pattern formation and development of metazoan animals. Misregulation of Hedgehog signaling has also been associated with the formation of multiple types of cancer. For these reasons, the Hedgehog pathway has attracted considerable interest. Many proteins that are required in the Hedgehog pathway have been identified, and while much has been learned about their function in signal transduction it is clear that this complement of proteins does not comprise the full set necessary for Hedgehog signal transduction. Because significant gaps remain in our knowledge of the molecules required for Hedgehog signaling we performed an enhancer/suppressor screen in *Drosophila melanogaster* to identify novel components of the pathway. In addition to the isolation of new alleles of the known pathway components *patched* and *smoothened*, this screen isolated fourteen novel complementation groups and a larger number of loci represented by single alleles. These groups include mutations in the genes encoding the translation factors eRF1 and eIF1A, and the kinesin-like protein Pavarotti. It also identified mutations in a gene whose product is necessary for the movement of Hedgehog protein through tissues.
Introduction

The Hedgehog (Hh) proteins are secreted morphogens that provide positional information during the development of many multicellular animals. Hh was originally identified as a segment polarity gene in *Drosophila* where it is required for the patterning of the embryonic cuticle. It has since been found to be involved in many other developmental processes including the patterning of adult legs, eyes, and wings. In vertebrates, Hh signaling is known to function in the patterning of many different structures, including the fore brain, neural tube, somites, eye and limb. Misregulation of Hh signaling has been implicated in basal cell carcinomas, gliomas, and gastric and prostate cancers (for reviews see (INGHAM and MCMAHON 2001; RUIZ I ALTABA et al. 2002).

One of the defining characteristics of morphogens, such as Hh, is their ability to elicit different responses in different cells in a concentration dependent manner (TABATA and TAKEI 2004). In the *Drosophila* wing imaginal disc, Hh is produced by cells in the posterior compartment and moves into the anterior compartment forming a concentration gradient. Anterior cells close to the anterior/posterior (A/P) compartment boundary see high levels of Hh and express both high threshold genes, such as *engrailed* (*en*) and *patched* (*ptc*), and low threshold genes such as *decapentaplegic* (*dpp*). Further from the A/P boundary the levels of Hh are insufficient to generate the high threshold response and only low threshold genes are expressed (see (BROOK 2000).

The ability of Hh to induce differential responses can be partially explained by the dual activity of the zinc finger transcription factor Cubitus-interruptus (Ci), the downstream nuclear effector of the Hh pathway (METHOT and BASLER 1999). In the
absence of Hh signaling the full-length Ci protein (Ci-155) is sequestered in the cytoplasm by a multi-protein complex containing the kinesin-like protein Costal-2 (Cos2), the serine/threonine kinase Fused (Fu) and the novel protein Suppressor of fused [Su(fu)] (Jia et al. 2003; Lum et al. 2003; Ogden et al. 2003; Robbins et al. 1997; Ruel et al. 2003; Sisson et al. 1997). This complex targets Ci for serial phosphorylation by at least three kinases; Protein kinase A (PKA), Glycogen synthase kinase 3β, (GSK3 β) and Casein kinase 1α (CK1 α) (Chen et al. 1998b; Jia et al. 2002; Price and Kalderon 1999; Price and Kalderon 2002). This phosphorylation targets Ci for ubiquitination by the Slimb/SCF complex (Jiang and Struhl 1998; Ou et al. 2002; Theodosiou et al. 1998). Ubiquitinated Ci then undergoes a proteosome dependent cleavage to generate a smaller (Ci-75) repressor form of the protein that translocates to the nucleus and constitutively represses the expression of Hh target genes (Aza-Blanc et al. 1997).

Smootherned (Smo) is a 7-pass membrane protein that is essential for all Hh signaling (Alcedo et al. 1996; Chen and Struhl 1996; van den Heuvel and Ingham 1996). The activity of Smo is repressed by the Hh-binding, multi-pass transmembrane receptor Patched (Ptc), since in the absence of Ptc downstream signaling events are activated by Smo in a ligand independent manner (Chen and Struhl 1996; Hooper 1994; Quirk et al. 1997). The binding of Hh to Ptc relieves the repression that Ptc normally exerts on Smo. The mechanism by which Ptc inhibits Smo activity is apparently not mediated by sequestration (Denef et al. 2000), and may involve an amplification step (Taipale et al. 2002), but the actual mechanism of repression remains unknown. In unstimulated cells Smo resides largely in intracellular vesicles, where it binds the Cos2 complex through a direct interaction between its cytoplasmic C-terminal tail and the Cos2
protein (JIA et al. 2003; LUM et al. 2003; OGDEN et al. 2003; RUEL et al. 2003; ZHU et al. 2003). Although the nature of Smo activation is not well understood, it is known that in response to Hh, Smo becomes hyperphosphorylated, is stabilized and translocates to the cell surface (DENEF et al. 2000), bringing along with it the Cos2 complex. These events are correlated with the stabilization of full-length Ci-155 and a concomitant loss of repressor Ci-75 (ALCEDO et al. 2000; DENEF et al. 2000; LUM et al. 2003).

In wing imaginal discs the de-repression of Hh target genes caused by the stabilization of Ci-155 is sufficient for the expression of dpp and other low threshold target genes (METHOT and BASLER 1999). The expression of high threshold genes, however, requires the conversion of Ci-155 into a transcriptional activator and its translocation into the nucleus (METHOT and BASLER 2000). This activation of Ci-155 is Hh dependent and requires Smo protein. The mechanism of Ci-155 activation is poorly understood, but likely involves relieving the repressive effects that Su(fu) has on Ci-155 (METHOT and BASLER 2000; WANG et al. 2000).

Many gaps remain in our understanding of Hh signaling. Ptc has homology to bacterial proton-driven transmembrane molecular transporters, and it has been proposed that Ptc functions by moving across the plasma membrane a small molecule that regulates Smo function (TAIPALE et al. 2002). It is not yet known what the small molecule is, or how it regulates the activity of Smo, or, for that matter, what actually is Smo activity. Recent reports have connected Smo with downstream components of the pathway by demonstrating a direct interaction between Smo and Cos2, and have shown that Smo phosphorylation and altered subcellular localization are correlated with pathway
activation. But it remains unclear how these interactions and activities result in the stabilization of Ci-155, or the conversion of Ci155 into a potent transcriptional activator.

Many questions also remain as to how the Hh concentration gradient is formed and shaped. The Hh protein undergoes several processing events, and the mature signaling form contains two different lipid additions. A cholesterol group is added to the C-terminal end of the protein during the autocatalytic cleavage of Hh (BUMCROT et al. 1995; LEE et al. 1994; PORTER et al. 1995), and an acyl group is added near the N-terminus by the transmembrane acyltransferase Sightless (CHAMOUN et al. 2001; LEE and TREISMAN 2001; MICCHELLI et al. 2002). The expression of the sterol sensing domain protein Dispatched in Hh producing cells, but not Hh receiving cells, is essential for the movement of mature Hh proteins from Hh producing cells into the Hh receiving tissue (BURKE et al. 1999). In contrast, there is a requirement for proteins involved in GAG biosynthesis (with acetylglucosaminyltransferase activity) in Hh receiving cells, but not Hh producing cells, which has led to the idea that heparin sulfate proteoglycans (HSPGs) are necessary for the movement Hh proteins in receiving tissues (HAN et al. 2004; TAKEI et al. 2004; THE et al. 1999). However, the mechanism by which lipid modified Hh proteins move through tissues, and the role of HSPG in this process, remains unknown.

These unresolved questions about Hh signaling and morphogen movement suggest that there are additional components to the pathway that have yet to be identified. To identify novel proteins required for Hh signaling we conducted a large-scale, genetic screen in Drosophila. In this screen we tested the ability of newly induced mutations to enhance or suppress of a partial Hh loss-of-function phenotype generated by the transgenic expression of dominant negative form of Smo in the developing wing. In
addition to new alleles of known components of the Hh pathway 105 interacting mutations were identified, of which 34 are grouped into 14 novel complementation groups. The isolation and genetic characterization of these mutations are described here.

**Materials and Methods**

**Molecular Biology**

The UAS-Smo5A was constructed using standard molecular biology techniques. The Smo coding sequence was mutated by PCR mediated mutagenesis to generate the mutations: TC2237GC, T2287G, AG2368GC, A2501A and A2539G with the corresponding amino acid changes: S667A, S687A, S740A, T755A and T785A (base pair and acid numbers according to accession number: NM_078719). The sequence: GACTACAAGGACGAGCAG was added immediately before the stop codon adding a FLAG epitope (DYKDDDDK) to the C-terminal end of the encoded protein. The resulting construct was ligated into the Not I and Xho I sites of the *Drosophila* cloning and transformation vector pUAST.

**Mutagenesis**

Isogenized *w*¹¹¹⁸ males were starved for 8 hours and then added to vials containing filters soaked with 25mM EMS (ethylmethanesulfonate, Sigma) and 1% sucrose in water. Males were mutagenized overnight, allowed to recover for 24 hours, and then crossed to C756-Gal4,UAS-Smo5A/TM3, HS-*hid* virgins. F1 flies with enhancement or suppression of the C756-Gal4,UAS-Smo5A (C765-SmoDN) phenotype were crossed to *Bl/Cy;C756-Gal4,UAS-Smo5A/TM6b* flies. F2 flies with the same enhancement or
suppression of the C765-SmoDN phenotype as the F1 fly were crossed again to 
Bl/CyO;C756-Gal4, UAS-Smo5A/TM6b. After the enhancement or suppression of the 
C765-SmoDN phenotype was confirmed again in the F3 generation males and females of 
genotype mutation/CyO;TM6b were crossed to establish balanced mutant stocks.

The rate of mutagenesis was estimated by dividing the total number of F1 TM3-
HS-hid flies by the number of ebony/TM3-HS-hid flies (i.e. the number of new 
mutations in the ebony gene). This gave a rate of one new ebony mutation every 1,600 F1 
genomes, or approximately 8 loss-of-function mutation per F1 genome. If one quarter of 
the genes in Drosophila are essential (BRIZUELA et al. 1994), then the mutation rate for 
the screen is about 2 lethal mutations per F1 genome.

Fly Stocks and Genetics

Fly stocks used included: w¹¹¹⁸, pavB²⁰⁰, ptcG¹², smo³, sit¹³⁹⁸, disp⁰³⁷⁷⁰⁷, hhAC, 
ptcG¹², smo³, Pka-C¹⁰¹²⁷₂, ttv⁰⁶⁶¹, Cos²⁴¹, eRF¹ neo²⁸, eRF¹ U³, mirr⁰¹⁰, mirr₄, Ptp6⁹D¹, 
Ptp6⁹D⁷, Ptp6⁹D¹⁸, C76⁵-Gal⁴, EP(3)0⁹³⁵, EP(3)3¹⁲¹, EP(3)3³⁵⁰, Df(3R)DG⁴, 
Df(3R)Cha¹, Df(3L)XS⁵₃₃, Df(3L)iro-², Df(3L)GN¹⁹, Df(3L)GN⁵⁰ (FLYBASE 2003), 
pav+¹¹⁰ (pav rescue construct, RC1; (ADAMS et al. 1998). Df(3L)ED⁴⁷¹⁰, 
Df(3L)ED²²⁴, Df(3L)ED²²⁵, Df(3L)ED⁴⁷⁸², Df(3L)ED⁴⁷⁹⁹, Df(3L)ED²²⁹, 
Df(3L)ED⁴⁸⁵⁸, Df(3L)ED⁴⁸⁶¹, Df(3L)ED⁴⁹⁷⁸ and Df(3L)ED²³⁰ (RYDER et al. 2004) 
and third chromosome deficiencies from the Bloomington Stock Center deficiency kit.

jaft mutant clones were generated by crossing males of genotype 
w; FRT8⁰,jai⁴⁴⁷/TM6B or w;dpp-LacZ; FRT8⁰,jai⁴⁴⁷/TM6B with females of genotype 
hs-flp; FRT8⁰, Ubi-GFP. Larvae were heat-shocked for one hour at 37 °C during the first 
and second instar to induce meiotic recombination. Clones of cells expressing Smo5A
were generated by crossing \textit{hs-flip; actin>CD2>Gal4;UAS-GFP}\ flies with \textit{w};\textit{UAS-Smo5A}\ flies and heat-shocking the larvae at 37° for 30 minutes.

To rescue \textit{pavarotti}\ mutants \textit{flies of genotype pav}^{+/10.5} /\textit{CyO};pav^{831}/TM6b or \textit{pav}^{+/10.5} /\textit{CyO};pav^{2046}/TM6b \ were crossed to \textit{flies of genotype pav}^{B200}/TM6b, \textit{pav}^{831}/TM6b, pav^{963}/TM6b or \textit{pav}^{2046}/TM6b.

\textbf{Dissections and immunohistochemistry}

Wing imaginal discs from climbing, third instar larvae were dissected into cold PBS and then fixed for 20 minutes at room temperature with 4% formaldehyde in PBS. Discs were then washed three times for 10 minutes with PBT (PBS with 0.2% Triton X-100), blocked for 45 minutes with BBT (PBT with 0.1% Bovine Serum Albumin) and incubated overnight at 4° in primary antibodies. Primary antibodies and dilutions used were anti-Dll (1:250; (WU and COHEN 1999), anti-phospho-Mad (1:1000; (PERSSON \textit{et al.} 1998), anti-Ci (Mab 2A1,1:2; (MOTZNY and HOLMGREN 1995), mouse anti-Ptc (1:3; (CAPDEVILA \textit{et al.} 1994) and rabbit anti-b-Gal (1:500; Kappel). After four, 30-minute washes at room temperature in BBT, discs were incubated for two hours in appropriate fluorescent-labeled secondary antibodies diluted 1:200 in BBT. Discs were washed four times with PBT and mounted in 80% glycerol in PBS for analysis with a Leica confocal microscope.

To mount adult wings, flies were incubated in \textit{SH buffer (20\% glycerol, 80\% ethanol)} overnight. After a rinse with water the wings were dissected into water and then mounted in Faure’s mounting media.
Results

Smo5A acts as a dominant negative

Previous work has demonstrated that Smo becomes phosphorylated in response to Hh and that this phosphorylation is correlated with Hh signal transduction (Deneff et al. 2000). A search of the primary structure of the Smo with PROSITE identified five putative protein kinase A (PKA) phosphorylation sites in the C-terminal, cytoplasmic tail (red boxes in figure 1A). To test if these sites are important for Smo function, transgenic flies were generated to express a mutant protein in which the target serines and threonines have been replaced with alanines (UAS-Smo5A see materials and methods). Clones of cells in the wing imaginal disc expressing the mutant Smo5A protein and GFP failed to express the Hh target Ptc (arrows in figure 1B and C), suggesting that Smo5A protein has dominant negative activity.

Expression of UAS-Smo5A throughout the developing wing imaginal disc with the C765-Gal4 driver caused a reduction in the distance between veins III-IV and a partial fusion of these veins proximal to the anterior cross vein (compare figure 1E with 1D). Hh signaling directly specifies the vein III-IV intervein region, and the UAS-Smo5A,C765-Gal4 phenotype (hereafter referred to as the C765-SmoDN phenotype) is consistent with a partial loss of Hh signaling during wing development. Indeed, reducing the dosage of Smo by one half resulted in strong enhancement in the C765-SmoDN phenotype (figure 1F). Conversely, reducing repression of the pathway by removal of one copy of ptc resulted in nearly normal wings (figure 1G).

A modifier screen for novel components of the Hedgehog pathway
The expression of UAS-Smo5A with C765-Gal4 produces a mild Hh loss of function phenotype, primarily in the wing of flies that are otherwise viable and fertile. The C765-SmoDN phenotype is visible in adult flies under the dissecting microscope, very consistent from individual to individual, and readily modified by reducing the dosage of genes encoding components of the Hh pathway. Furthermore, the C765-SmoDN phenotype is not significantly modified in flies heterozygous for components of other signaling pathways necessary for wing development (e.g. Wg or Dpp; data not shown). Because of these characteristics, we used the C765-SmoDN phenotype as sensitized background in a F1 enhancer/suppressor screen to identify novel components of the Hh pathway.

In the screen isogenic w^{1118} males were mutagenized with EMS and crossed to females carrying the UAS-Smo5A and C765-Gal4 transgenes (the crossing scheme is summarized in figure 2). The progeny of these flies were scored for enhancement or suppression of the C765-SmoDN phenotype. F1 flies with modifying mutations were crossed back to flies with UAS-Smo5A and C765-Gal4 transgenes and second and third chromosome balancers. Male progeny of backcrosses that confirmed the original modification were again backcrossed. This second back-cross permitted the establishment of balanced mutant stocks, mapped the interacting mutation to the second or third chromosome, and confirmed the modification of the C765-SmoDN phenotype in a third generation.

A total of approximately 90,000 genomes were screened, and 2,558 F1 flies were selected as having potentially interesting mutations. After the two backcrosses, balanced
stocks had been established for 107 mutants that showed a consistent and penetrant modification of the C765-SmoDN phenotype.

The balanced mutations were tested for complementation with genes for known components of the Hh pathway, including $sit^{T398}$, $disp^{so370}$, $hh^{AC}$, $ptc^{G12}$, $smo^{3}$, $Pka-C1^{01272}$, $ttv^{00681}$ and $Cos2^{W1}$. New alleles of $ptc$ ($ptc^{1232}$) and $smo$ ($smo^{848}$) were isolated, demonstrating that the screening strategy was effective in finding mutations in genes required for Hh signaling.

The remaining mutations were tested for cross-complementation with the other mutations that mapped to the same chromosome. These complementation crosses placed 34 of the mutations into 14 different lethal complementation groups, two on the second chromosome and 12 on the third chromosome (Table 1). Remarkably, one mutant fly contained mutations for two separate complementation groups (B-left and B-right). These mutations are separable by recombination, each enhances the C765-SmoDN phenotype, and each complements members of one group and fails to complement members of the other. The remaining 71 mutations represent single hits.

We next sought to identify the genes disrupted by the mutations. Mapping mutations is greatly simplified when there are multiple alleles, and most of our complementation groups are on the third chromosome. We have therefore concentrated our efforts on mapping and characterizing these groups.

The 12 lethal complementation groups on the third chromosome were mapped with several techniques including standard meiotic recombination onto a chromosome with multiple recessive markers ($rucuca$), complementation crosses with deficiencies, P element-mediated meiotic recombination mapping (Zhai et al. 2003), and P element-
mediated male recombination mapping (Chen et al. 1998a). The mapping results for the third chromosome groups are summarized in figure 3 and, for several groups, discussed in detail in the following sections. The interaction between each of the groups and C765-Gal4,UAS-Smo5A is shown in figure 4.

To better study the function of the complementation groups in Hh signaling, individual mutations were recombined onto FRT chromosome, and clones of mutant cells were generated by somatic recombination (Harrison and Perrimon 1993). Both alleles of Group E are heterozygous fertile, however recombinants of either allele onto the FRT80 chromosome appear to be male sterile. It has, therefore, not been possible to generate FRT stocks for Group E. No mutant clones were recovered for eight of the 12 groups (Groups A, B-left, B-right, D, I, J, K and M, see table 1) even though wild type twin spots, with two copies of the clonal marker, were present in adult eyes. This suggests that the mutations in these groups are cell lethal.

While clones of cells that are mutant for Group G do not seem to persist to the adult, these cells can survive long enough to occasionally have dramatic effects on wing and eye development. Some flies in which clones have been induced have scars in the eye/missing photoreceptors, rough eyes, wing blisters, wing margin notching, and altered wing blade morphology (data not shown). No obvious phenotypes were observed in legs, halteres, antennae, thorax or abdomen. However, other flies with distinct twin spots in the eye appeared morphologically normal.

**Translation factors eIF1A and eRF1**

Group I (comprising two members: $I^{654}$ and $I^{2232}$) was mapped by meiotic recombination between the rucuca markers stripe and ebony. Complementation crosses
with deficiencies in the region demonstrated that both Group I alleles failed to complement Df(3R)Cha7 (90F01-F04;91F05), but complimented two overlapping deficiencies; Df(3R)DG4 (90D02-04;90F03-06) and Df(3R)Cha1a (91A02-B03;91F13-92A01). This defined an interval for the Group I region of approximately 425 Kb between the genes *stripe* and *fruitless*. Testing for complementation with lethal P insertions in the region revealed that the P insertions EP(3)0935 and EP(3)3350 failed to complement both alleles of group I. Both P insertions are located in the gene encoding the translation elongation factor eIF-1A (LASKO 2000; PENA-RANGEL et al. 2002) suggesting that Group I is allelic with *eIF-1A*.

Group J (comprising three members: J^{709}, J^{2604} and J^{2633}) mapped between the markers *scarlet* and *curled* by meiotic recombination. Group J members failed to complement Df(3L)rdgC-co2 (77A01;77D01) but complemented the overlapping deficiencies Df(3L)XS533 (76B04;77B) and Df(3L)ri-79c (77B-C;77F-78A). This placed Group J in the interval between 77B and 77C. Complementation crosses with lethal P insertions in the interval identified one insertion that failed to complement Group J. This P element, EP(3)3121 is inserted in the gene encoding the elongation release factor eRF1 (ABDELILAH-SEYFRIED et al. 2001), suggesting that the Group J alleles are mutations in this gene. Indeed, a second P insertion in the eRF1 locus (eRF1^{neo28}) and an EMS allele (eRF1^{U3}) both failed to complement the Group J mutations.

**The Iroquois complex gene mirrored in Group F**

Two lethal enhancer mutations (F^{1486} and F^{1825}) comprise Group F. These mutations mapped between the recessive markers *roughoid* (61F) and *thread* (72C) by meiotic recombination. Testing for complementation with deficiencies in the region
identified three overlapping deficiencies that failed to complement both group F mutations: Df(3L)eygC1 (69A4-5:69D4-6), Df(3L)BSC10 (69D4-5;69F5-7) and Df(3L)iro-2 (69B1-5;69D1-6). This suggests that the gene mutated in Group F is in an interval between 69D4 and 69D6. We tested for complementation with mutations of known genes in the region and found that both alleles of Group F failed to complement mutations in the mirror gene (mirr<sup>Cre2</sup> and mirr<sup>SaiD1</sup>; (KEHL et al. 1998; MCNEILL et al. 1997).

The Group F mutations also failed to complement Ptp69D<sup>i</sup>. However, this allele of Protein tyrosine phosphatase 69D was generated by the local transposition of the mirr<sup>Cre2</sup> P insertion into the Ptp69D locus, followed imprecise excision (DESAI et al. 1996). mirr<sup>Cre2</sup> fails to complement the lethality of Ptp69D<sup>i</sup>, whereas it is complemented by mirr<sup>SaiD1</sup>, suggesting that a lethal mutation in mirr is retained in the Ptp69D<sup>i</sup> stock. It is therefore most likely that the Group F mutations fail to complement the Ptp69D<sup>i</sup> stock because of the second lethal mutation in mirr and not the mutation in Ptp69D and thus the Group F mutations are new alleles of mirr.

**Group D are mutations in the gene encoding the kinesin like protein Pavarotti**

The three members of Group D comprise a single lethal complementation group, but interestingly D<sup>831</sup> and D<sup>2046</sup> were identified as enhancers whereas D<sup>963</sup> was identified as a suppressor. Group D mapped between roughoid and hairy by meiotic recombination and deficiencies in this area were tested for complementation. Df(3L)GN24 (63F06-07;64C13-15) and Df(3L)GN50 (63E01-02;64B17) failed to complement the Group D mutations, whereas the overlapping deficiencies Df(3L)ZN47 (64C; 65C) and Df(3L)GN19 (63F04-07;64B09-11) complemented. This defined an interval of
approximately 895 Kb between the genes ImpL2 (uncovered by Df(3L)GN19; (Garbe et al. 1993) and Srp54K (uncovered by Df(3L)ZN47; (FlyBase 2003) that contains Group D.

To further refine the position of the Group D mutations, a mapping technique of measuring the recombination distance between the mutations and molecularly defined P element insertions was used (Zhai et al. 2003). Of the 20,412 recombination events scored, only three occurred between Group D and the EP(3)1135 insertion. This places Group D at a distance of 0.015 centiMorgans from this P insertion (see figure 5A for details). Lethal mutations and insertions in nearby genes were tested for complementation. All three Group D members failed to complement a mutation in pavarotti (pav\textsuperscript{B200}), suggesting that the Group D mutants are alleles of the pav gene. To confirm this we tested if the lethality of the Group D mutations could be rescued by the pav rescue construct RC1 (Adams et al. 1998). A single copy of the RC1 transgene rescued to viability D\textsuperscript{831} and D\textsuperscript{2046} when transheterozygous with pav\textsuperscript{B200}. D\textsuperscript{963} was also rescued by RC1, both when homozygous or in trans with the other Group D alleles. However, the RC1 transgene did not rescue the lethality of either D\textsuperscript{831} or D\textsuperscript{2046} when homozygous, or the D\textsuperscript{831}/D\textsuperscript{2046} transheterozygous combination. pav\textsuperscript{B20} enhances the C765-SmoDN phenotype but not as strongly as D\textsuperscript{831} or D\textsuperscript{2046} (compare figure 5E with B and C). This suggests that pav\textsuperscript{B200} may be hypomorphic and that D\textsuperscript{831} or D\textsuperscript{2046} are stronger, possibly null alleles. This could explain the failure of the RC1 construct to rescue the homozygous lethality of D\textsuperscript{831} or D\textsuperscript{2046}. 
Group C disrupts a novel gene required for Hh movement

With four members (C^{447}, C^{477}, C^{789} and C^{2075}), Group C is the largest complementation group identified in the screen. It has been mapped by meiotic recombination between FRT80B and *scarlet* (73A) on chromosome 3L. All of the deficiencies in the third chromosome deficiency kit (as provided by the Bloomington stock center) that map to this region complement Group C. The DrosDel (RYDER *et al.* 2004) deletions; Df(3L)ED4710 (74D1;75B11), Df(3L)ED224 (75B2;75C6), Df(3L)ED225 (75C1;77E6), Df(3L)ED4782 (75F2;76A1), Df(3L)ED4799 (76A1;76B3) Df(3L)ED229 (76A1;76E1), Df(3L)ED4858 (76D3;77C1), Df(3L)ED4861 (76F1;77E6), Df(3L)ED4978 (78D5;79A2) and Df(3L)ED230 (79C2;80A4) also complemented the Group C mutation.

In wing imaginal discs, Hh is expressed in the posterior compartment and the expression of Cubitus-interruptus (Ci), an essential downstream effector, is restricted to the anterior compartment. Hh protein produced by posterior compartment cells moves into the anterior compartment, creating an activity gradient that extends into the anterior compartment from the A/P compartment boundary. In the absence of Hh, the full-length Ci protein (Ci-155) is degraded into a smaller, repressor form (Ci-75). The activation of Hh signaling inhibits this degradation and induces the stabilization and accumulation of Ci-155 in anterior cells along the A/P compartment boundary (BROOK 2000). The up-regulation Ci-155 can be detected with the rat monoclonal anti 2A1 (see figures 6A, C, D and F) whose epitope is cleaved from the Ci-75 protein (MOTZNY and HOLMGREN 1995).

Clones of cells in the anterior compartment that are mutant for Group C (absence of green GFP clonal marker in figures 6A, B, D and E) fail to up regulate full-length Ci
(red in figures 6A, C, D and F) suggesting that the Group C gene product is essential for Hh signaling. Interestingly, Ci is up regulated normally in anterior cells opposite large clones of cells in the posterior compartment that abut the A/P boundary (Figure D-F). Therefore, Group C is dispensable in Hh producing cells for proper Hh signaling in wing discs.

Although most cells in anterior compartment clones fail to up-regulate Ci-155, mutant cells adjacent to the clone border closest to the source of Hh do accumulate full-length Ci (figure 6A-C). This non-autonomous effect can be seen more easily by examining the expression of decapentaplegic (dpp), a transcriptional target of Hh signaling, using a dpp-lacZ transgene. In wild type discs dpp-lacZ is expressed in a stripe approximately 10 cells wide along the anterior side of the A/P compartment boundary (red in figure 6G). This expression of dpp-lacZ is lost in clones of cells mutant for Group C, except for a single row of mutant cells along the border of the clone closest to the Hh source (figure 6H and I). Furthermore, wild type cells on the opposite side, or “downstream”, of the Hh source also fail to up-regulate Ci-155 (arrow in figures 6B and C). Thus, cells that are mutant for Group C are competent to respond to, and transduce, the Hh signal, but do not permit the movement of Hh protein. Therefore, the Hh response is absent from cells in the interior of the clones, and in wild type cells downstream of clones, because these cells never see the Hh signal.

The phenotypes described above are nearly identical to those caused by clones of cells mutant for the tout-velu genes (tout-velu (ttv), brother of tout-velu (botv) and sister of tout-velu (sotv); (HAN et al. 2004; TAKEI et al. 2004). We have therefore named the Group C gene jaf't, for just another tout-velu. ttv genes encode
acetylglucosaminyltransferases and are required for HSPG biosynthesis. HSPGs are thought to be required for the movement of Hh, as well as the movement of other signaling molecules such as Wg and Dpp (Han et al. 2004; Takei et al. 2004).

Clones of cells in wing discs that are mutant for ttv, botv or sotv severely disrupt Dpp signaling, and have more subtle effects on Wg signaling. We tested if clones of jatf would induce similar disruptions to these pathways. Dpp is expressed in a stripe of cells along the anterior side of the A/P boundary. The secreted Dpp protein moves away from these producing cells to form a gradient in both the anterior and posterior compartments (Entchev et al. 2000; Lecuit et al. 1996; Nellen et al. 1996; Teleman and Cohen 2000). The activity of the Dpp pathway can be detected using a phospho-specific antibody against the Mothers against dpp protein (Mad), a down stream component that becomes phosphorylated in response to Dpp signaling (Persson et al. 1998). Phosphorylated Mad (P-Mad) is undetectable in jatf mutant clones (arrows in figure 7B and C) except in a single row of cells closest to the source of Dpp (red cells indicated with arrows in figure 7A).

Wg is expressed along the D/V boundary and forms a concentration gradient in both dorsal and ventral compartments where it activates the expression of target genes such as Distal-less (Dll) (Neumann and Cohen 1997; Strigini and Cohen 2000; Zecca et al. 1996). Clones of cells mutant for jatf cause a reduction in the expression of Dll expression, especially in wild type cells downstream from the clone (arrow in figure 7F). Large clones of mutant cells reduce the range of Dll expression, and often have a more sharply defined border between expressing and non expressing cells than does wild type tissue (compare the broad, graded expressing of Dll in the posterior/ventral region with
the narrower, defined expression in the clone in the anterior/ventral region in figures 7D-F). The disruption of signaling caused by jaft mutant clones is nearly identical to those reported for clones of cells mutant for ttv, botv and sotv, suggesting that jaft may likewise encode a protein required for HSPG biosynthesis.

**Discussion**

We set out to identify novel components of the Hh signaling pathway by screening newly induced mutations for the ability to enhance or suppress a partial Hh loss of function phenotype in the *Drosophila* wing. In addition to new alleles of known components of the Hh pathway, *ptc* 1232 and *smo* 848, 105 novel interacting mutations were isolated. 36 of these mutations can be grouped into 14 lethal complementation groups.

Although numerous screens have been conducted to identify Hh signaling components, we isolated mutations in a large number of genes not identified in these previous screens. Interestingly, it was not possible to recover clones of mutant cells in either adult or larval imaginal discs for at least eight of our complementation groups, suggesting that these mutations are cell lethal. The pleiotropic effects of these mutations would likely prevent the isolation of these genes in genetic screens, except in an enhancer/suppressor screen such as the one presented here. Therefore, the genes identified in this screen may be important, novel mediators of Hh signaling. However, the cell lethality of many of the mutations makes it difficult to confirm or further examine the role of the encoded proteins in Hh signaling.

Two of the cell lethal complementation groups are mutations in genes encoding proteins essential for translation. The Group I mutations, *eIF1A* 645 and *eIF1A* 2232, are allelic with the gene encoding the eukaryotic Initiation Factor 1A (eIF1A) which is
required the stable association of the 40S complex with the 5’ cap (PestoVA et al. 2001). The three Group J mutations, eRF1\textsuperscript{709}, eRF1\textsuperscript{2604} and eRF1\textsuperscript{2633}, are new alleles of the eukaryotic Release Factor 1 (eRF1) gene. eRF1 is necessary for recognition of the stop codon and the termination of protein synthesis (Kisselev and Buckingham 2000).

It is possible that reducing the dosage of eIF1A or eRF1 diminishes the capacity of a cell to translate proteins. Because Hh signaling requires new translation of Smo protein (AJeDo et al. 2000; DeneF et al. 2000) and the downstream effects of Hh likely requires the synthesis of new proteins, a generalized reduction in protein translation could cause an enhancement of the C765-SmoDN phenotype. However, the Drosophila Minute (M) mutations are generally thought to be mutations in genes encoding ribosomal proteins, and flies heterozygous for M mutations have reduced protein synthesis. These flies present stereotypical, haploinsufficient phenotypes including delayed development, small body size, and small, thin bristles (Lambertsson 1998). None of the 107 mutants isolated in this screen display these phenotypes. Thus, M mutations were apparently not selected as enhancers of the C765-SmoDN phenotype, whereas two alleles of eIF1A and three alleles of eRF1 were isolated. This suggests that there is a specific interaction between these genes and Hh signaling, and raise the possibility that Hh signaling may directly promote protein translation.

Although Hh signaling is best known for its role in pattern formation it has also been shown to promote growth during normal development (Marti and BoVoleNTA 2002; Ruiz i AlTaba et al. 2002) and in tumorigenesis (Berman et al. 2003; Thayer et al. 2003; Watkins et al. 2003). In wing imaginal discs Hh signaling is necessary for the growth of the central, vein III-IV intervein region (Mullor et al. 1997; Strigini and
COHEN 1997). It has been reported that this growth is mediated by the helix-loop-helix transcription factor encoded by the Hh target gene knot (CROZATIER et al. 2002), but Hh may also promote growth by increasing translation of new proteins through the up-regulation eIF1A and eRF1. eIF1A has been shown to be up-regulated in response to hyper activation of JAK/STAT signaling (MYRICK and DEAROLF 2000), however it is not known if increasing levels of eIF1A promotes protein translation. The potential regulation of eIF1A and eRF1 expression by Hh signaling, and its possible role in tissue growth awaits further experimentation.

Two new alleles of mirror, mirr\textsuperscript{1486} and mirr\textsuperscript{1825}, were identified as enhancers of the C765-SmoDN phenotype. mirr is a member of the Iroquois complex and encodes a homeodomain transcription factor (KEHL et al. 1998; MCNEILL et al. 1997). The other members of the Iroquois complex, araucan and caupolican, are transcriptional targets of Hh signaling in the wing imaginal disc, and are necessary for the patterning of the veins and sensory organs (GOMEZ-SKARMETA and MODOLELL 1996). The isolation of mirr mutations suggests that mirr may also be a target of Hh signaling. However, in third instar discs expression mirr was not detected in the central portion of the wing pouch (KEHL et al. 1998), the region of the wing disc where the genetic interaction with Hh was observed. It is possible that mirr is expressed there, but below detectable levels. Alternatively, mirr may be expressed in this central region of the wing pouch during earlier stages of development and disruption of this earlier expression results in the enhancement of the C765-SmoDN phenotype.

The mutations in Group D, pav\textsuperscript{831}, pav\textsuperscript{963}, and pav\textsuperscript{2046} are new alleles of Pavarotti (pav), a gene encoding a kinesin-like protein. Pav has been shown to required for the
organization of central spindle and contractile ring, and cytokinesis fails in cells lacking Pav (ADAMS et al. 1998). Although the role of pav in cytokinesis has been studied in some detail no function for Pav in Hh signaling has yet been demonstrated, and pav was not identified in previous screens for Hh pathway components. However, cells lacking Pav are unable to complete cytokinesis and the resulting multi-nucleated cells are cleared by apoptosis. This nearly cell-lethal phenotype would likely mask any role of Pav in Hh signaling, and prevent its isolation in genetic screens other than a modifier screen such as this.

In response to Hh signaling Ptc moves from the cell surface to intracellular vesicles, and Smo moves from intracellular vesicles in the cytoplasm to the plasma membrane. This movement of Smo to the cell surface is correlated with signal transduction, and both the movement of Smo and Ptc requires the actin cytoskeleton and the microtubule network (JIA et al. 2003; LUM et al. 2003; ZHU et al. 2003). It is possible that Pav mediates the movement of Smo, Ptc, or both, in response to Hh signaling. Alternatively, Pav may function to move or maintain Ci-155 in the nucleus of Hh responding cells, as Pav is primarily nuclear localized during interphase. However, expression of a mutant Pav protein that is unable to localize to the nucleus is able to rescue pav mutants to adulthood (MINESTRINI et al. 2003), suggesting that Pav nuclear localization is not essential for cytokinesis or Hh signaling.

The four members of Group C, jaft<sup>447</sup>, jaft<sup>477</sup>, jaft<sup>789</sup>, and jaft<sup>2075</sup>, are mutations in a novel gene we have named jaft. Clones of cells mutant for jaft in the wing imaginal disc are defective in Hh signaling. The phenotypes caused by clones of mutant cells indicate that in the absence of jaft cells can still produce the Hh signal, and can respond to the
signal if they see the Hh protein. However, Hh protein is unable to move through the \textit{jaft} mutant tissue, causing a loss of Hh signaling in the interior of mutant clones and in wild type cells “downstream” of the Hh source. The movement of Dpp and Wg proteins are similarly disrupted in these clones, although Wg movement is disrupted to a much less extent than either Hh or Dpp.

These phenotypes are identical to those caused by clones of cells mutant for \textit{tout-velu, brother of tout-velu, or sister of tout-velu. ttv, botv and sotv} encode proteins with acetylglucosaminyltransferase activity and are required for the biosynthesis of HSPGs (\textit{Han et al.} 2004; \textit{Takei et al.} 2004). It is the phenotypes induced by mutations in these genes that have demonstrated the requirement for HSPGs for the movement of Hh and other morphogens. But what HSPGs do that facilitates the movement of signaling molecules is not yet known.

Because mutations of \textit{jaft} induce phenotypes that are identical to those caused by mutations of the \textit{tout-velu} like genes, it is likely that the \textit{jaft} gene product is also required for HSPG biosynthesis. Mapping of \textit{jaft} mutations has placed the gene between 73A and 80B on chromosome 3L. None of the genes that reside in this interval encode obvious HSPG biosynthetic proteins. Therefore, \textit{jaft} may encode a protein that plays a regulatory or modulatory role in HSPG biosynthesis, and the cloning and further characterization of \textit{jaft} should provide novel insight into the role of HSPGs in morphogen movement.

\textbf{Acknowledgements}

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### TABLE 1

**Screen Summary**

<table>
<thead>
<tr>
<th>Cross</th>
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<tbody>
<tr>
<td>F1 Flies Screened</td>
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<tr>
<td>F1 Enhancer/Suppressor Selected</td>
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<tr>
<td>Balanced Mutant Stocks</td>
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<table>
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<tr>
<th>Complementation Groups</th>
<th>Chromosome</th>
<th>Number of Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>smoothened</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>patched</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Group N</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Group O</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Group A*</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group B-left*</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Group B-right*</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Group C (jaft)</td>
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<td>4</td>
</tr>
<tr>
<td>Group D (pavarotti) †</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Group E</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Group F (mirror)</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
Group G‡         3
Group I (eIF1A)*         2
Group J (eRF1)*         3
Group K*         2
Group M*         2

Single Hits         71

* No clones of mutant cells recovered in adults or third instar wing discs, possibly cell-lethal.
† Required for cytokinesis, clones of mutant cells not recovered in adults or third instar wing discs.
‡ Clones of mutant cells were not recovered in adults or third instar wing discs, but persist long enough to occasionally induce phenotypes in wing and eyes (see text).
Figure 1. Smo5A is a dominant negative. A) Primary sequence of smoothened, signal sequence (grey), transmembrane domains (green) C-terminal cytoplasmic tail (blue) and five putative PKA phosphorylation sites (red) that were mutated to alanines are highlighted. Expression of Smo5A in flip-out clones (green GFP expressing cells in B) represses the expression of Patched expression (red; B and C). Expression of Smo5A in the wing with the C765-Gal4 (E) driver causes a reduction of the central portion of the wing between longitudinal veins III and IV and fusion of these veins proximal to the anterior cross vein (compare to wild type; D). These phenotypes are strongly enhanced in flies heterozygous for smo (F), and are completely suppressed by removing one copy of ptc. Longitudinal veins I-V and the anterior cross vein (ACV) are indicated in D.

Figure 2. Crossing scheme for the screen. EMS mutagenized males were crossed to females with the C765-Gal4,UAS-Smo5A tester chromosome (C5). The F1 progeny with enhancement or suppression of the C5 phenotype were crossed to the backcross strain (containing the C5 chromosome, the second chromosome dominant marker Bristle, and second and third chromosome balancers). F2 flies that showed penetrant modification of the C5 phenotype were again crossed to the backcross strain to generate a balanced mutant stock, and to map the modifying mutation to the second or third chromosome.

Figure 3. Summary of mapping complementation groups on the third chromosome. The cytological map positions or intervals the third chromosome complementation groups are indicated by bars and open boxes respectively.
**Figure 4.** Modification of the C765-Gal4,UAS-Smo5A by third chromosome complementation groups. The twelve complementation groups on the third chromosome enhance the C5 phenotype (see figure 1E for comparison). The group and mutant number are indicated.

**Figure 5.** Group D mutations are alleles of pavarotti. A) The cytological positions, and the distance in centiMorgans from Group D, of the insertions used for P element mediated meiotic recombination mapping are indicated on the upper line. The region around EP(3)1135, which is only 0.015 cM from Group D, is shown in the lower line. pav is approximately 10 kB from the EP(3)1135 insertion site. Mutations in pav modify the C5 phenotype. Two alleles of pav identified in the screen, pav^{831} and pav^{2046}, enhance the C5 phenotype (B and D respectively) where as the third allele, pav^{963}, suppresses the C5 phenotype (C). The previously described pav^{B200} allele also enhances the C5 phenotype (E).

**Figure 6.** Group C is required for Hedgehog signaling. Confocal images of third instar wing imaginal discs containing clones of cells mutant for Group C (loss of green GFP expression in A,B,D,E and H) and stained for full-length Ci protein (red in A,C,D and F) or anti-b-Gal (red in G-I) to show expression of dpp-LacZ. In wild-type tissue, Ci-155 is stabilized by Hh signaling, this results in a broad band of stronger staining cells on the posterior side of the A/P boundary (arrows in C indicate the width of Ci up-regulation in wild-type tissue). Clones of cells mutant for group C fail to up-regulate Ci-155 (A, C) except, notably, those cells that are immediately adjacent to the Hh source. Also, wild-
type cells on the opposite side of clone from the source of Hh non-autonomously fail to
up-regulate Ci-155. Ci-155 is up-regulated normally in posterior cells (arrow in F) when
these cells are adjacent to a large, anterior clone at the A/P boundary (loss of green GFP
expression), suggesting that Group C in not required in Hh producing cells. In wild-type
wing imaginal discs dpp-lacZ is expressed in a stripe along the posterior side of the A/P
boundary (red in G). In clones of cells for group C (loss of green GFP expression in H)
dpp-lacZ expression (red in H and I) lost, except in a single row of cells immediately
adjacent to the A/P boundary and the Hh source.

Figure 7. Wg and Dpp signaling are disrupted in jaft mutants. Dpp is expressed
along the A/P compartment boundary and signals in a graded manner in both anterior and
posterior compartments. Pathway activation can be detected with antibodies against the
phosphorylated form of the downstream component Mad (P-Mad, red in A and C). In
clones of cell mutant for jaft (absence of green GFP expression in A and B) there is no
detectable phosphorylated Mad (arrows in B and C). Wg secreted from the D/V margin
activated the graded expression of the Dll target gene in both dorsal and ventral
compartments (red in D and F). In clones of cells mutant for jaft (absence of green GFP
expression in D and E) only those cells closest to the Wg source express Dll, and in jaft
mutant clones Dll expression ends in a sharp border rather than the graded expression in
wild-type tissue.
Collins and Cohen, Figure 1
Collins and Cohen, Figure 2

P  
\[\text{♂ EMS treated, isogenic } w^{T16} \]  
\[\times \]  
\[\text{♀ } C765-\text{Gal}4,\text{UAS-Smo}5\text{A} \]  
\[\text{TM3-Hshid} \]

F1  
\[\text{♂ }  \text{Enricher/Suppressor} \]  
\[\text{♀ } C765-\text{Gal}4,\text{UAS-Smo}5\text{A} \]  
\[\times \]  
\[\text{♀ } C765-\text{Gal}4,\text{UAS-Smo}5\text{A} \]  
\[\text{CyO ; TM6B} \]

F2  
\[\text{♂ }  \text{Enricher/Suppressor} \]  
\[\text{♀ } C765-\text{Gal}4,\text{UAS-Smo}5\text{A} \]  
\[\times \]  
\[\text{♀ } C765-\text{Gal}4,\text{UAS-Smo}5\text{A} \]  
\[\text{CyO ; TM6B} \]

F3  
\[\text{♂ }  \text{Enricher/Suppressor} \]  
\[\text{♀ } \text{CyO ; TM6B} \]

Figure 3

3L  
61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98  99  100

J (pRF1)

D (poly) | G | F (minor) | K | B-left | C (inf)

3R  
82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98  99  100

B-right | M | l (eIF-1A) | A
Collins and Cohen, Figure 4
Collins and Cohen, Figure 6
Collins and Cohen, Figure 7