The *shavenoid* gene of *Drosophila* encodes a novel actin cytoskeleton interacting protein that promotes wing hair morphogenesis.

Nan Ren, Biao He *, David Stone, Sreenatha Kirakodu ** and Paul N. Adler

Biology Department, Cancer Center and Morphogenesis and Regenerative Medicine Institute, University of Virginia, Charlottesville VA 22903.

* current address: Department of Surgery, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0104

** current address: Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, KY 40536
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Corresponding author:

Paul Adler
Biology Department
Gilmer Hall, rm 245
University of Virginia, Charlottesville, VA 22903
E-mail: pna@virginia.edu
Telephone 434-982-5475
FAX 434-982-5626
ABSTRACT

The simple cellular composition and array of distally pointing hairs has made the Drosophila wing a favored system for studying planar polarity and the coordination of cellular and tissue level morphogenesis. The developing hairs are filled with F-actin and microtubules and the activity of these cytoskeletons is important for hair morphogenesis. Based on mutant phenotypes several genes have been identified as playing a key role in stimulating hair formation. Mutations in \textit{shavenoid} (\textit{sha}) (also known as \textit{kojak}) result in a delay in hair morphogenesis and in some cells forming no hair and others several small hairs. We report here the molecular identification and characterization of the \textit{shavenoid} (\textit{sha}) gene and protein. \textit{sha} encodes a large novel protein that has homologs in other insects, but not in more distantly related organisms. The Sha protein accumulated in growing hairs and bristles in a pattern that suggested it could be directly interacting with the actin cytoskeleton. Consistent with this mechanism of action we found that Sha and actin co-immunoprecipitated from wing disc cells. The morphogenesis of the hair involves temporal control by \textit{sha} and spatial control by the genes of the \textit{frizzled} planar polarity pathway. We found a strong genetic interaction between mutations in these genes consistent with their having a close but parallel functional relationship.
The Drosophila wing has been one the prime model systems for studying morphogenesis at both the cellular and tissue levels. The wing is the largest Drosophila appendage and a great deal has been learned about the genetic basis for wing patterning and the regulation of wing cell proliferation (e.g. (De Celis 2003; Edgar 1999; Martin et al. 2004; Teleman and Cohen 2000). In addition, the flat simple structure of both the pupal and adult cuticular wing has made it a favored system for studies on cellular morphogenesis and planar polarity (Adler 2002; Eaton 2003). Most wing blade cells differentiate a single distally pointing cuticular hair. The extension that forms the hair contains both actin filaments and microtubules and the function of both cytoskeletons is required for normal morphogenesis (Eaton et al. 1996; Turner and Adler 1998; Wong and Adler 1993). The distal polarity of hairs is regulated by the frizzled (fz) tissue polarity pathway (Wong and Adler 1993). The timing of hair initiation is at least indirectly regulated by the ecdysone cascade, but relatively little is known about how temporal aspects of wing cell differentiation are controlled. Among the genes previously implicated as having a role in regulating the time of hair initiation are grainy head (Lee and Adler 2004), sha (sha - is also known as kojak) (He and Adler 2002) and ovo/svb (Delon et al. 2003). Mutations in sha and ovo/svb often lead to the failure of a cell to form a hair.

Mutations in sha also affect the differentiation of two additional types of extensions of epidermal cells. The long thin laterals found on the arista (the distal most segment of the antenna) are the product of single epidermal cells and in a sha mutant the laterals are both branched, multiplied and shorter than normal (He and Adler 2002). In vivo observation of the development of laterals in sha mutants revealed that lateral initiation was delayed about 6 hours and the subsequent growth was also slower than normal. EM thin sections showed that the
distribution of actin filament bundles was abnormal in mutant laterals. *sha* mutations also result in a reduction in the number of larval denticles and those that are present are shorter and thinner than normal (Nusslein-Volhard et al. 1984). Interestingly *sha* does not display a mutant phenotype in sensory bristles, which share many characteristics with arista laterals (He and Adler 2002).

We report here the molecular characterization of the *sha* gene and protein. Previous work in our lab had mapped *sha* to it to a 60 kb region in 47F (He 2001). In a separate study of gene expression in pupal wings we identified one annotated gene in this region (CG13209) whose expression increased 11 fold from 24 to 32 hours suggesting it could be *sha* (Ren et al. 2005). We confirmed this by identifying the sequence changes associated with 6 EMS/gamma-ray induced *sha* alleles, by identifying a P insertion allele and by transformation rescue. Somewhat surprisingly we found that the even expression of *sha* from a transgene was sufficient to rescue the mutant phenotype, thus the temporal change in expression level was not essential. The *sha* gene encodes a 179 kd protein that is conserved in other insects. We found the Sha protein accumulated close to the plasma membrane in growing hairs suggesting it functions directly in the hair to promote cytoskeletal mediated outgrowth. When expressed in bristles the Sha protein appeared to localize between the large bundles of actin filaments found in these cells and the plasma membrane. We further found that Sha and actin could be co-immunoprecipitated from wing disc cells consistent with Sha acting directly on the cytoskeleton. To determine if Sha was sufficient to activate the cytoskeleton to initiate hair morphogenesis we examined the effects of driving *sha* expression at other developmental stages. We failed to see any effects of *sha* expression on the actin cytoskeleton in third instar wing discs or in young pupal wings. Hence, *sha* is necessary but not sufficient to activate the cytoskeleton to drive hair morphogenesis.
formation. We also found strong genetic interactions between mutations in *sha* and mutations in genes of the *frizzled* planar polarity pathway consistent with these two systems respectively controlling temporal and spatial aspects of hair morphogenesis.

### MATERIALS AND METHODS

**Fly stocks:** *FRT, FLP, GFP* expressing, mutant and deficiency carrying chromosomes were obtained from the Drosophila Stock Center in Bloomington. The *sha* gene is also known as *kojak* (*koj*). In this paper we use *sha*.

**Clonal Analysis:** Somatic clones were generated using the FRT/FLP system (Xu and Rubin 1993). Pupal wing clones were marked by the loss of GFP. Unmarked clones were detected by mutant phenotypes.

**Cytological techniques:** White prepupae were collected and aged until dissection. Immunostaining was done by standard techniques after fixation with paraformaldehyde (He et al. 2005). Fluorescent secondary antibodies and fluorescent phalloidin for staining the actin cytoskeleton were obtained from Molecular Probes. Confocal images were obtained on an ATTO CARV confocal unit attached to a Nikon microscope. *In situ* hybridization on pupal wings was done as described previously using digoxygenin labeled probes (Geng et al. 2000).

**Generating antibodies:** A 516 bp fragment that encodes the amino terminal 172 amino acids of Sha was subcloned into the pET28a vector. Expression of fusion protein was induced with IPTG and the fusion protein was purified using the his6 tag provided by the vector. Fusion protein was injected into rats and guinea pigs at Spring Valley Laboratories, Inc. (Sykesville,
MD). The sera was used without further purification.

**Immunoprecipitation and Western Blotting:** For western blotting 20 third instar wing discs were dissected in cold PBS and homogenized in SDS sample buffer. The extract was heated at 100°C for 5 minutes and then 8% SDS-PAGE gels (Invitrogen) and blotted with Millipore Immobilon-P transfer membrane (Sigma). The blot was probed with the desired primary antibody and detected using Supersignal West Pico reagents (Pierce).

For immunoprecipitation experiments twenty to forty wing discs were dissected from third instar larvae and homogenized in pre-chilled lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 5mM EDTA). The extract was spun at 12,000g at 2-8 ºC for 15 min to remove cellular debris. To reduce the background caused by non-specific adsorption of irrelevant cellular proteins, a preclearing step was done by adding protein A agarose beads (Roche) to the sample and incubating at 2-8 ºC for 3 hours. The beads were pelleted by centrifuged at 12,000g for 30 seconds. Ten μl of anti-Sha antibody was added to the supernatants, the samples were incubated at 2-8 ºC for 2 hours, then added to 50 μl of protein A agarose (Roche). The samples were rotated gently at 2-8 ºC overnight. Beads were then washed three times with washing buffer 1 (50mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 protease inhibitor tablet from Roche), wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Nonidet P40, 0.05% sodium deoxycholate) and wash buffer 3 (10 mM Tris-HCl, pH 7.5, 0.1% Nonidet P40, 0.05% sodium deoxycholate), treated with protein sample/gel-loading buffer (Sigma) and denatured proteins by heating to 100 ºC for 3 minutes. The protein samples were stored at –20 ºC and then analyzed by western blotting as noted above.

**Fractionation of the Sha protein:** Wing discs were dissected from Drosophila third instar larvae and homogenized in pre-chilled lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM
NaCl, 5mM EDTA) and the extract was spun at 500 g for 10 min by micro-centrifuge to remove particulate materials. The supernatant was again spun at 16,000 g for 10 min, and the soluble fraction (S1) and the pellet fraction (P1) were analyzed by western blotting.

**Construction of the sha cDNA:** As noted in the text the BDGP did not recover a *sha* cDNA clone so we made one using RT-PCR. Because of the length of the *sha* ORF the amplification was done in 2 pieces. One fragment covered exons 1-5 and the other exons 5-8. The 5' fragment was inserted into pBluescript as a Not1-HindIII fragment. The Not 1 site was put into the primer and the Hind III site is present in the endogenous gene. The 3' fragment was subcloned into this fragment using the Hind III site and an added Kpn 1 site resulting in a complete cDNA. The appropriate NotI-KpnI fragment 4881 bp sha coding region was inserted into the pUAST vector (Brand and Perrimon, 1993). The GFP tag was inserted as a Kpn I – Xba I fragment into sha-pUAST.

**RESULTS**

**The sha mutant phenotype:** As described previously *sha* mutations lead to some wing cells forming no hair, others forming multiple small hairs and some cells forming relatively normal hairs (Figure 1) (HE and ADLER 2002). The severity of the phenotype varied across the wing. In general the phenotype was stronger on the ventral vs dorsal surface and in medial and proximal wing regions than in peripheral and distal regions. The regions with the most severe phenotypes were ones where hair initiation occurred last, suggesting a connection between the time of hair initiation and the mutant phenotype. Even in null alleles rare cells formed relatively
normal hairs, thus *sha* was not absolutely essential for building a hair. In this way it differed from genes such as *crinkled (ck)*, which encodes a cellular myosin as all hairs in a *ck* mutant showed a mutant phenotype (KIEHART et al. 2004; TURNER and ADLER 1998). The epidermal hair phenotype of *sha* was not restricted to the wing and was seen in all regions of the adult epidermis. The strength of the phenotype varied from one tissue to another. It was strong on the notum (dorsal thorax) (Figure 2, A,B) and legs (data not shown), which lost essentially all hairs and weaker on the abdomen, where hairs were lost in a patchy pattern. (Figure 2, C,D). The *sha* mutant phenotype was not restricted to the adult epidermis and was also seen in larvae, where it caused a loss and reduction in size of denticles (Figure 2, E,F).

In genetic mosaics *sha* acted largely cell autonomously. Neighboring wild type cells differentiated normally and mutant cells at the clone edges were not rescued (Figure 3). However, the mutant phenotype of *sha* was enhanced in mutant clones relative to entirely mutant wings (Figure 1, D,E). Thus, most mutant cells in clones failed to produce any hair, even when located in wing regions that only showed a weak phenotype in an entirely mutant wing. This did not appear to be correlated with clone size and was true even for very small clones. We suggest this apparent lack of perdurance was due to the dramatic increase in *sha* expression just prior to hair initiation. The enhancement of the mutant phenotype in clones indicated that in some way neighboring wild type cells functioned to inhibit the differentiation of mutant clone cells.

**Molecular identification and characterization of *sha*:** Genetic screens in this laboratory resulted in the isolation of 18 *sha* alleles. Four of these were on chromosomes that had a rearrangement in the 47F region. RFLPs on these chromosomes mapped to over more than 50 kb and a dozen genes and did not pin point the *sha* transcription unit (HE 2001). It is likely
that multiple hits were generated during the mutagen treatment. We found the expression of one gene in this region CG13209 increased 11 fold from 24 to 32 hours AWP (REN et al. 2005). We sequenced CG13209 from 6 cytologically normal sha mutations (and a parental chromosome for 3 of these). We identified mutations associated with all of these alleles (Figure 4). Three of the alleles were missense mutations. One of these was the temperature sensitive hypomorphic shaVB13 allele. This resulted from a mutation of pro 195 to leu. This proline is conserved in all of the insect species where we have identified sha homologs and is in the center of a highly conserved region (Figure 4). A second missense mutation was the strong shaVAI51 allele that resulted from a change of arg 314 to ser. This residue is also conserved in all insect species where we have identified sha homologs (Figure 4). The third missense mutation was the strong shaXO51 allele that resulted in a change of asn 446 to lys. This asn is conserved in all of the dipteran sha genes (Figure 4). Two of the strong alleles were associated with nonsense mutations in CG13209 (Figure 4). One (shaVAG11) was predicted to produce a 378 amino acid protein and the other (shaVE41) a 966 amino acid protein. The sixth allele was associated with a single base pair insertion that results in 8 new amino acids being added after amino acid 1260 change followed by a termination codon (Figure 4). Interestingly this allele (shaFK2871) acted as a weak dominant negative (Figure 1, G,H)). While these experiments were on going we became aware that the BDGP had isolated a P insertion approximately 1 kb upstream of CG13209 that was a semi-lethal (KG08508). We found that the mutant escapers were phenotypically sha. The shaP allele failed to complement members of our collection of sha alleles consistent with it being an insertion into sha. We confirmed that the insertion was responsible for the sha mutation as the mobilization of the P insert reverted the mutation.
The BDGP predicted that *CG13209* encoded a 4881 nucleotide mRNA from 8 exons. However, no *sha* cDNA clone was recovered by the BDGP leaving some doubt about the accuracy of the transcript prediction. We confirmed the existence of all of the predicted splice sites by sequencing RT-PCR products (data not shown). We constructed a *sha* cDNA that contained all of the predicted coding sequences, fused it to GFP, subcloned this into pUAST and generated transgenic flies. When the expression of this transgene was driven by *act-GAL4* it provided almost complete *sha* rescue activity in the wing (Figure 1, C,F,I) and arista (data not shown) (a few cells in the rescued wings produced small hairs). Thus, the expression of the predicted Sha-GFP fusion protein was sufficient for essentially normal *sha* function in the two tissues where it had principally been studied.

Clones homozygous for *sha* mutations showed a cell autonomous wing hair phenotype in all regions of the wing suggesting that *sha* was expressed and functioned in all wing cells. We examined the expression of *sha* by *in situ* hybridization and found as expected that it was expressed at relatively even levels across the wing (Figure 5E).

It is worth noting that the dramatic increase in *sha* expression seen in our gene chip experiments did not appear to be essential for *sha* function as *act-Gal4* is expected to drive transgene expression in a relatively uniform fashion. To determine if *sha* expression was sufficient to stimulate hair initiation we examined *ptc-GAL4 UAS-sha-GFP* pupal wings from 24 - 32 hours after white pupae (Figure 6, G,H,I). We did not see any signs of premature hair initiation nor any abnormal accumulation of F-actin. We also failed to see any effect on the actin cytoskeleton of third instar *ptc-GAL4 UAS-sha-GFP* wing discs (data not shown). We concluded that *sha* was not sufficient to activate the cytoskeleton. Perhaps Sha needed to act with another protein to stimulate hair outgrowth.
We examined the sequence of sha homologs in other Drosophila species where genome sequence is available (http://species.flybase.net/). The intron exon structure of sha is conserved in the melanogaster subgroup. The 5th exon in *D. melanogaster* is split into 2 exons in *D. mojavensis* and *D. virilis* and the the 8th exon in *D. melanogaster* is split into 2 exons in *D. virilis*.

**The Sha protein:** Conceptual translation of sha yielded a protein of 1626 amino acids with a predicted molecular weight of 179,311 Da. The protein did not contain any informative motifs. Some, but not all programs tested predicted a single transmembrane domain. To determine if Sha was an integral membrane protein we homogenized Sha expressing wing discs in a low salt buffer in the absence of any detergent. The Sha protein, as assayed by western blotting, was soluble under these conditions arguing that it is not an integral membrane protein (Figure 5C).

*sha* has clear homologs in other Drosophila species. In species such as *D. erecta*, *D. virilis*, *D. yakuba* and *D. pseudoobscura* the protein is well conserved over essentially the entire sequence. In comparisons between members of the melanogaster subgroup we found 96% identity and 97% similarity. In more distantly related species such as *D. virilis* we found 73% identity and 80% similarity to *D. melanogaster sha*. Homologs of *sha* can also be found in other insects. In *Anopheles gambiae* and *Aedes aegypti* we could construct conceptual homologs that were conserved over more than 1400 amino acids. The percent identity fell to 33% and 30% and the percent similarity to 46% and 41% respectively. We could also identify regions of similarity in other insects such as *Bombyx mori*, *Apis mellifera* and *Tribolium castaneum*, although for these organisms we could find homology to at most about 950 amino acids of *sha*.
The percent identity here ranged from 44% to 38%. That these identities were higher than those seen for the mosquitoes was likely due to only the most highly conserved regions being identified. The completion of the sequencing and annotation of their genomes will be needed to determine if these animals contain homologs that contain most if not all of *D. melanogaster sha*. We did not find any homologs in non-insect species. Using the reiterative psi-blast program (Altschul *et al.* 1997) we detected weak similarity to a number of proteins in more distantly related organisms. These included the *C. elegans* UNC89, which encodes a very large protein that is required for myofibril organization in *C. elegans*. This protein contains a myosin light chain kinase domain (Benjan *et al.* 1996; Small *et al.* 2004).

**Sha accumulates in developing hairs:** We took two approaches to examine the subcellular distribution of the Sha protein, both of which gave similar results. In one we examined the distribution of the transgene encoded Sha-GFP fusion protein in pupal wing cells taking advantage of the GFP tag. We found the *sha* protein in developing hairs consistent with it acting at the level of the cytoskeleton (Figure 6, B,C). In developing hairs Sha-GFP appeared to accumulate to somewhat higher levels proximally than distally. In hairs viewed perpendicular to the long axis we could see that Sha was peripheral (Fig. 6C) suggesting it could be mediating the attachment of cytoskeletal elements to the plasma membrane. The peripheral localization of Sha often appeared more extensive on the distal side of the hair. In developing bristles we observed Sha-GFP in longitudinal stripes (Figure 6, A,D-F). In bristles stained for both Sha and actin these stripes of Sha were largely co-localized with the large actin bundles that are juxtaposed to the plasma membrane in bristles. The co-localization was not complete as the Sha protein routinely appeared to be more peripheral than the actin (Figure 6E) consistent with Sha connecting the actin bundles to the plasma membrane.
As an alternative approach we generated an anti-Sha antibody. When we stained pupal wings using this antibody we also found anti-Sha staining in the developing hairs although the background was often high (data not shown). Only weak staining (essentially background) was detected prior to hair formation as expected from the expression profile of the gene. As a control we examined pupal wings that contained sha clones and found a loss of staining in the clones (Figure 6 J-L). We examined the accumulation of Sha using our polyclonal antibody to probe western blots of pupal wing proteins. As a first test of the usefulness of this antibody for western blots, we examined extracts of whole wild type larvae and larvae where Sha expression was driven by Act-GAL4. A strong high molecular weight (>170 Kd) signal was detected in transgene driven extracts (Figure 5A). We also examined extracts of 24 and 32 hr pupal wings. We did not detect any sha protein in the 24 hr sample but a signal was detected in the 32 hr sample (Figure 5B) consistent with the marked increase in sha RNA levels seen.

The location of the Sha protein next to the plasma membrane and actin bundles at sites of hair and bristle outgrowth suggested the hypothesis that sha functioned in hair development by interacting directly with one or more components of the actin cytoskeleton to promote actin polymerization and hair outgrowth. To test this possibility we immunoprecipitated sha protein from wing discs and then analyzed the precipitate by western blotting. We found that actin was co-immunoprecipitated with Sha consistent with the possibility that it interacted directly with the actin cytoskeleton (Figure 5D).

sha with planar polarity genes: In experiments where we used sha as a cuticular marker in mosaic experiments with planar polarity genes we generated chromosomes that were mutant for sha and planar polarity genes such as pk, Vang, and stan (Adler et al. 2000; Chae et al. 1999; Gubb et al. 1999; Taylor et al. 1998; Tree et al. 2002; Usui et al. 1999; Wolff and
To maximize the viability of the mutant animals we used the hypomorphic $sha^{VB13}$ allele in many of these experiments. Because of the enhanced $sha$ phenotype seen in clones even a weak allele serves as a reliable cuticular marker. Doubly mutant animals that carried the hypomorphic $sha^{VB}$ allele showed a dramatically enhanced $sha$ mutant phenotype (Figure 7, Table 1). All of the planar polarity mutants we examined ($fz$, $in$, $fy$, $frtz$, $pk$, $Vang$, $stan$ and $dsh$) (AXELROD 2001; CHAE et al. 1999; SHIMADA et al. 2001; TAYLOR et al. 1998) (COLLIER and GUBB 1997; COLLIER et al. 2005; GUBB et al. 1999; PARK et al. 1996; TREE et al. 2002; VINSON and ADLER 1987) acted as enhancers (Figure 7, C,E,F). In the stereomicroscope the adult flies had bowed, wet looking wings. In the compound microscope we could see that the doubly mutant animals had large wing regions where cells formed no hair. In this way the phenotype was substantially stronger than that of a null $sha$ allele (Figure 1). For several of the double mutant combinations we quantified the fraction of the wing where cells formed no or only a very small hair and this increased dramatically in the double mutants (Table 1). We also examined flies that were doubly mutant for a $fz$ pathway mutation and a null $sha$ allele (Figure 7D). The null $sha$ phenotype was also enhanced in these wings but it was difficult to quantify as most of the wings were at least partially folded and difficult to score (Figure 7, B,D). Since the phenotype of a null $sha$ allele was enhanced we can rule out the possibility that the $fz$ pathway functions upstream of $sha$. In regions where hairs formed the polarity and multiple hair cell phenotypes of the $fz$ pathway mutants did not seem to be altered (data not shown).

Based on observations that suggested the $fz$ pathway regulated the site for hair initiation and that $sha$ regulated the timing of hair initiation (HE and ADLER 2002; WONG and ADLER 1993) it seemed likely that the 2 pathways functioned in parallel. The observations noted above argued that the $fz$ pathway did not function upstream of $sha$. To test if $sha$ could be functioning
upstream of the planar polarity genes we examined the distribution of two planar polarity proteins (Stan/Fmi and In) in sha clones in pupal wings. The Stan/Fmi protein accumulates at both the proximal and distal sides of wing cells (Usui et al. 1999) and In accumulates at the proximal side (Adler et al. 2004). stan function is required for the function and asymmetric accumulation of all of the planar polarity proteins, while in functions as a downstream component of the fz pathway. Both proteins accumulated asymmetrically in sha clones indicating that sha function is not required for fz pathway function (Figure 3, D-F – data for in not shown).

DISCUSSION

sha – a key player in hair morphogenesis: Mutations in sha produce perhaps the most dramatic wing hair phenotype of any known Drosophila gene – loss of the hair. Previously we found that sha mutations resulted in a delay in both wing hair and arista lateral initiation (He and Adler 2002). Temperature shift experiments with the temperature sensitive shaVB13 allele were consistent with sha principally functioning at or just prior to hair and lateral initiation (He and Adler 2002). Based on these observations we suspected that sha expression might be linked to hair initiation. We found the expression of CG13209 sharply increased from 24 to 32 hrs (Ren et al. 2005) and combined with previous mapping data it suggested that sha could be CG13209. We showed this was correct both by identifying sequence changes associated with 6 sha alleles and transformation rescue. It is interesting that the ubiquitous expression of sha provided rescue activity even though the expression of the endogenous sha gene is strongly modulated in vivo. It appears that sha is essential but not instructive with respect to hair initiation. Consistent with
this hypothesis we saw no evidence for an alteration in the actin cytoskeleton nor an effect on the timing of hair initiation from premature expression ofsha. In principlesha could produce its dramatic mutant phenotype by interacting either directly or indirectly to activate either the actin or microtubule cytoskeletons. Direct activation could entail providing a site on the membrane for the polymerization of actin while indirect activation could work by regulating the expression of hair forming genes. Consistent withsha playing a direct role in hair outgrowth we found the Sha protein accumulated at the periphery of growing hairs and we found that Sha and actin co-immunoprecipitated from wing disc cells. When expressed in developing bristles we found that Sha accumulated in longitudinal stripes that largely co-localized with the large bundles of actin filaments seen in these cells. These large bundles are in close association with the plasma membrane and Sha appeared to be displaced slightly on the peripheral side of the actin bundles. This suggests that Sha is localized on the membrane. Based on these observations we suggest that Sha serves to activate/organize actin polymerization by recruiting it to the plasma membrane. Developing arista laterals contain actin bundles that are similar to but smaller than those seen in bristles. Thin sections of mutantsha laterals showed abnormal actin bundles (HE and Adler 2002). The bundles were irregular in shape and often not juxtaposed to the plasma membrane consistent with our hypothesis that Sha regulates/organizes actin at the plasma membrane.

The Sha protein sequence does not provide any “smoking guns” as to Sha biochemical function. However, a weak similarity was detected to several proteins that are known to interact with the actin cytoskeleton (e.g. UNC89) consistent with our finding that Sha and actin can be co-immunoprecipitated from disc cells. This interaction suggests that mechanisticallysha acts directly on the actin cytoskeleton to promote hair morphogenesis. In vitro studies will be needed
to determine the biochemical function of sha. For example, does Sha promote actin polymerization or bundling? Unfortunately Sha is rather large, which makes in vitro studies difficult.

**sha functions in parallel to planar polarity genes:** The \( fz \) planar polarity pathway regulates hair morphogenesis in at least two ways. It controls polarity by controlling the subcellular location for hair initiation a process that requires downstream planar polarity effectors such as Inturned, Fuzzy and Frtz (Adler et al. 2004; Collier et al. 2005; Lee and Adler 2002). The \( fz \) planar polarity pathway also activates the cytoskeleton for hair morphogenesis via RhoA mediated Rho kinase activation of Spaghetti Squash (Drosophila myosin regulatory light chain) (Winter et al. 2001). We found that mutations in \( fz \) pathway genes act as strong enhancers of the \( sha \) mutant hair phenotype. This enhancement is seen with null \( sha \) alleles thus the \( fz \) pathway appears to act in parallel to \( sha \). This implies that the regulation of the location and timing of hair initiation converge on a common target. This could be achieved either by there being a common target that integrates these inputs and then regulates the cytoskeleton or by these pathways independently regulating the cytoskeleton.

**Cell autonomy of sha:** By the classical genetic mosaic tests \( sha \) mutations act completely cell autonomously. All \( sha \) mutant cells show a mutant phenotype and all neighboring wild type cells differentiate normally. However, the phenotype of \( sha \) mutant cells in a clone is substantially stronger than is seen when the entire wing is mutant. Thus, in some way the presence of wild type cells influences the differentiation of the mutant clone cells. This could be due either to a diffusible signal from another tissue or to an influence by neighboring wild type cells in the wing epithelium. We suggest that this effect is a consequence of the delayed and slower morphogenesis of \( sha \) mutant cells (He and Adler 2002). When the entire
animal is mutant the process of metamorphosis may be delayed in a way that allows the mutant wing cells more time to build a hair. This could result in mutant cells being able to form small multiplied hairs. In a mosaic animal the overall timing of differentiation may be closer to normal. Mutant cells in a clone would have less time to elaborate a hair prior to an irreversible block to further hair morphogenesis. This could result in a cell that did not form any obvious hair. If this is the case what could be the nature of the “block”. Such a block could be hormonal and/or due to cuticle deposition and cross linking. Cuticle starts being secreted during the process of hair outgrowth (GUILD et al. 2005). Perhaps in an entirely mutant animal cuticle secretion and/or cross linking is delayed as well as other aspects of epidermal development. In sha clone cells the timing of cuticle deposition and/or cross linking could be driven by wild type cells elsewhere in the animal. The deposition and/or cross linking of cuticle prior to the outgrowth of the hair could prevent any subsequent delayed hair outgrowth by mutant cells.

Conservation of Cytoskeleton regulatory genes: Many genes that are important for development are highly conserved across a wide spectrum or organisms. This is true for many genes that play an important role in the morphogenesis of the array of hairs that cover the adult Drosophila epidermis. The elaboration of hairs involves the actin and microtubule cytoskeletons and many genes that play an important role in cytoskeleton function in other organisms have a similar role in Drosophila (BAUM 2002). Thus, mutations in numerous genes that encode conserved cytoskeleton interacting proteins result in strong wing hair phenotypes. A classic example of such a gene is singed which encodes a fascin that functions in bundling actin (BRYAN et al. 1993; CANT et al. 1994). The hairs of sn mutants are curved and bent. Mutations in genes that encode cytoskeleton regulatory proteins such as the Tricornered kinase that result in strong hair phenotypes are also often highly conserved (GENG et al. 2000; HE et al. 2005). Mutations in
tricornered give rise to wing cells that can form as many as a dozen small hairs instead of a single long one. Thus, it is somewhat surprising that sha is not conserved beyond the insects. Within the genus Drosophila sha is moderately well conserved. Drosophila melanogaster and Drosophila virilis are thought to have diverged 40 million years ago and the sha proteins from these two species remain 73% identical and 80% similar over 1663 amino acids. Compared to other genes known to be important for hair morphogenesis we find this is similar to pawn (72% identical and 78% similar), higher than inturned (63% identical and 75% similar and much lower than singed (99 % identical and 99.8% similar) or tricornered (97% identical and 98% similar) (ARRUDA and DOLPH 2003; BRYAN et al. 1993; CANT et al. 1994; GENG et al. 2000; PARK et al. 1996). Sha may be an example of a gene whose primary structure changes during evolution to facilitate morphological changes.

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Table 1
Planar Polarity genes enhance *shavenoid* hair phenotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fraction of dorsal wing showing strong <em>sha</em> phenotype (sd)</th>
<th>Comparison to <em>sha</em></th>
</tr>
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<tbody>
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<td><em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;</td>
<td>0.14 (0.003)</td>
<td>NR</td>
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<tr>
<td><em>fz</em>&lt;sup&gt;R54&lt;/sup&gt;</td>
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<td>NR</td>
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<tr>
<td><em>dsh</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.00</td>
<td>NR</td>
</tr>
<tr>
<td><em>in</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.00</td>
<td>NR</td>
</tr>
<tr>
<td><em>fritz</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.00</td>
<td>NR</td>
</tr>
<tr>
<td><em>Vang</em>&lt;sup&gt;TBS42&lt;/sup&gt;</td>
<td>0.00</td>
<td>NR</td>
</tr>
<tr>
<td><em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;; <em>fz</em>&lt;sup&gt;R54&lt;/sup&gt;</td>
<td>0.58 (0.06)</td>
<td>Enhance (p&lt;0.000001)</td>
</tr>
<tr>
<td><em>dsh</em>&lt;sup&gt;1&lt;/sup&gt;; <em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;</td>
<td>0.70 (0.01)</td>
<td>Enhance (p&lt;0.000001)</td>
</tr>
<tr>
<td><em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;; <em>in</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.55 (0.03)</td>
<td>Enhance (p&lt;0.000001)</td>
</tr>
<tr>
<td><em>fritz</em>&lt;sup&gt;2&lt;/sup&gt; <em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;</td>
<td>0.65 (0.04)</td>
<td>Enhance (p&lt;0.000001)</td>
</tr>
<tr>
<td><em>Vang</em>&lt;sup&gt;TBS42&lt;/sup&gt; <em>sha</em>&lt;sup&gt;VB13&lt;/sup&gt;</td>
<td>0.69 (0.01)</td>
<td>Enhance (p&lt;0.000001)</td>
</tr>
</tbody>
</table>

* a t test was used to compare the fraction of the wing surface having a strong phenotype (very small or no hairs).

NR – not relevant
Figure 1. *sha* adult wing phenotype. All micrographs are of adult wings and in all images proximal is to the left and distal to the right. Panels A, B, D, E, G and H are all equivalent images (same magnification and wing region (dorsal surface, center of the E region (most posterior region of the wing)) and panels CFI are a second set (ventral surface proximal part of the C cell (central region of the wing)). Panel A shows a wild type wing (Ore-R). Panel B shows a *sha*VAI51/Df wing (strong, phenotypic null allele). Almost all of the cells in this region of the *sha*VAI51/Df wing form several small hairs. Panel D a *sha*VB13/shaVB13 wing from an animal raised at 25°C. This allele is a temperature sensitive hypomorphic allele that has a very weak phenotype in the dorsal E region. The hairs are slightly finer and are more elevated, so that they appear shorter than they are. Panel E shows an unmarked *sha*VB13 clone in the dorsal E region. The asterisk shows the clone. Note that the clone cells do not produce a hair. Note how this phenotype is stronger than that in panel D even though the cells have the same genotype. This shows how enhanced the *sha* phenotype is in clones. Panel G shows a region from the dorsal E region from a fly that carried one copy of the dominant negative *sha*FK2871 allele. The arrow points to a cell that did not form a hair. Panel H shows the same region from a fly hemizygous for *sha*FK2871. Note the phenotype is stronger than seen with the phenotypic null (panel B). Panel C is from the ventral surface of a wild type wing (Ore-R). Panel F is from *sha*VB13/shaVB13. Note that in this region of the wing the phenotype is strong. Panel I is from
the same region of a $sha^{VB13}/sha^{VB13}$, $act$-Gal4 UAS-sha-GFP wing. Note the rescue by the transgene.

Figure 2. The $sha$ phenotype in other body regions. Panels A and B are from the dorsal notum (thorax) of wild type (Ore-R) (A) and $sha^{VAI51}/Df$ (B). Note that the bristles appear normal but the epidermal hairs are lost in the mutant. Panels C (Ore-R) and D ($sha^{VAI51}/Df$) are from the dorsal abdomen (4th abdominal segment). Once again note that the bristles are normal in the mutant but the small epidermal hairs are reduced. Panels E ($sha^{VAI51}/Df$) and F (Ore-R) are from the central region of the dorsal third abdominal segment from a third instar larvae. Note how the denticles are smaller and less numerous in the mutant.

Figure 3. The $sha$ mutant phenotype in pupal wings. Panels ABC show a $sha^{VAI51}$ clone in the wing marked by a loss of GFP (panel C). Panel A shows the wing hairs labeled by staining the actin cytoskeleton using Alexa 568 phalloidin. Panel B is the merged image. Note the lack of hair formation by the mutant cells. Panels DEF show a $sha^{VAI51}$ clone in the wing marked by a loss of GFP. The wing was stained using an anti-Starry night (aka flamingo) antibody. Note the typical zig zag pattern seen for planar polarity proteins and that this is not altered by the $sha^{VAI51}$ clone. Similar results were obtained for In (data not shown).

Figure 4. Molecular Characterization of $sha$. The top of the figure is a cartoon showing the $sha$ protein and the location of 6 different $sha$ alleles. Below are the amino acid sequences for insect shaak proteins surrounding the regions where the 3 missense mutations are located. The location
of the mutation is indicated by an asterisk. Residues that are identical to the Drosophila melanogaster sequence are in bold. Below are the consensus sequence for each 30 amino acid region. Residues that are completely conserved are in bold. Note that in several instances there appears to be a small deletion or insertion. We were unable to identify the region surrounding residue 445 in Tribolium.

Figure 5. Expression of sha. Panel A shows a western blot comparing act-Gal4 UAS sha-GFP larval and Oregon R larval extracts. The blot was probed with anti-Sha polyclonal antibody. Note the strong signal in the transgene driven lane. Panel B shows a western blot probed with anti-Sha antibody that compares extracts from 24 and 32 hr pupal wings. Note the signal present in the 32 hr sample that is missing in the 24 hr sample (arrow). The lower part of panels A and B show an unknown protein that was routinely detected by the secondary antibody on this set of Western blots and it serves as a loading control. Panel C shows a Western blot of act-Gal4 UAS sha-GFP wing disc proteins probed with anti-Sha antibody. The wing discs were homogenized in a low salt buffer. An aliquot of this is shown in the total lane. The sample was then centrifuged and the pellet and supernatant fractions applied to the gel. Note the Sha protein is soluble in low salt buffer. Panel D shows the results of an immunoprecipitation experiment. act-Gal4 UAS sha-GFP and Oregon R wings were homogenized and immunoprecipitated with anti-Sha antibody. As a control no antibody was added to one sample. The precipitated material was then assayed by a western blot using either anti-sha or anti-actin antibodies. Note the anti-Sha antibody was able to pull down actin. Panel E shows an in situ hybridization to 32 hr pupal wings using CG13209 antisense (upper) and sense (lower) probes. We conclude that sha is expressed in all regions of the pupal wing.
Figure 6. Sha protein accumulates in growing hairs and bristles. Panel A shows Sha-GFP in pupal head epidermis. Longitudinal stripes of GFP (arrow) are seen in developing bristles and epidermal hairs (arrowhead) also stain brightly. Panel B shows a section of a pupal wing expressing sha-GFP. The hairs stain brightly. Panel C shows a high magnification image of several hairs oriented perpendicular the the plane of focus. The peripheral staining of GFP can be seen and this is brighter on the distal side of the hair (arrow). Panels DEF are of bristles on the marginal row of the wing that express Sha:GFP (D - green) and are stained for actin (F – red). Panel E is the merged image. Note that the green staining extends beyond the red (arrow). Panels GHI show a 28 hr wing that expresses sha:GFP driven by ptc-GAL4. Actin is in red (G) and GFP in green (I) and the merge is shown in H. Note the expression of sha does not appear to influence the distribution of actin. Panels JKL show a sha clone in the wing marked by the loss of GFP. The pupal wing is stained with anti-sha antibody (red). Note the loss of Sha staining in the clone (asterisk) and the staining of the hairs outside of the clone (arrow).

Figure 7. sha interacts with the fz pathway. Panel A shows a region from the dorsal E region of the wing from a shaVB13 fly raised at 25°C. The phenotype is very weak in this region on such flies. Panels CE and F show VangTBS42 shaVB13, frtz2 shaVB13 and shaVB13; in double mutants respectively. Note how the sha hair loss phenotype is strongly enhanced by each of the planar polarity mutants. Panel B shows the same region from a shaVAI51/Df wing and panel F from a pk shaVAI51 double mutant. Note that the pk mutant enhanced the phenotype of the phenotypic sha null allele (shaVAI51).
**Shavenoid Protein**

0 500 1000 1500

**wt**

P R N N

**sha**

L 195

S 314

**sha**

K 446

**sha**

967

**sha**

378

**sha**

T 1261

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195 *--sha**

SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR Drosophila melanogaster
SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR Drosophila yakuba
SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR Drosophila erecta
SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR Drosophila pseudoobscura
SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR Drosophila virilis
TAPFIS GSS S-EKIPVF LP HRQI VHP SR Anopheles gambiae
TAPFIS GSS S-EKIPVF LP HRQI VHP SR Aedes aegypti
AATPCT GLS N-QUIPF VF LP LKGQIIHP SR Bombyx mori
NVAGIG QVERPVYF LP QRGQIIYP HA Apis melifera
MAPFVGST S-ETIPVF LP LKGQIIHP SR Tribolium castaneum

SLSPFVS GSS S-EKIPVF LP LKGQIIYPSR consensus

314 *--sha**

MPPRNVHSPC VAFRVNGSPV KYAHVPEVFF Drosophila melanogaster
MPPRNVHSPC VAFRVNGSPV KYSHVPEVFF Drosophila yakuba
MPPRNVHSPC VAFRVNGSPV KYASHVPEVFF Drosophila erecta
MPPRNVHSPC VAFRVNGSPV KYAHVPEVFF Drosophila pseudoobscura
MPPRNVHSPC VAFRVNGSPV KYAHVPEVFF Drosophila virilis
MNQNIFTPC IAFRVGTP I KVTVNTEV SF Anopheles gambiae
LVNTSKY INRIKLCY YSFLDVEVSOF Bombyx mori
L-GVFTPC VAFRVAGS QS KTSRYSEVTYF Apis melifera
PTGTYLF VAFRVG SPT K-F-DVEVAF Tribolium castaneum

MPPRNVHSPC VAFRVNGSPV KYAHVPEVFF consensus

446 *--sha**

RTSIGLKE D MGIVKNNPLL QHFPLS ER Drosophila melanogaster
RTSIGLKE D MGIVKNNPLL QHFPLS ER Drosophila yakuba
RTSIGLKE D MGIVKNNPLL QHFPLS ER Drosophila erecta
RTSIGLKE D MGIVKNNPLL QHFPLS ER Drosophila pseudoobscura
RTSIGLKE D MGIVKNNPLL QHFPLS ER Drosophila virilis
LANVGL HAE MGIVKNNPLL KHFPNLSD-- Anopheles gambiae
ALNVGL HTE MGIVKNNPLL KHFPNLSD-- Aedes aegypti
QSLPHMQRD DGMKKNPLL SN-----TY TD Bombyx mori
------------- GLV KS NPLL AASRF-ES D Apis melifera

RTSIGLKE D MGIVKNNPLL QHFPLS ER consensus