Title

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Journal

Insect Molecular Biology, 31(2)

ISSN

0962-1075

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Publication Date

2022-04-01

DOI

10.1111/imb.12752

Peer reviewed

HHS Public Access

Author manuscript

Insect Mol Biol. Author manuscript; available in PMC 2023 September 28.

Published in final edited form as:

Insect Mol Biol. 2022 April ; 31(2): 216–224. doi:10.1111/imb.12752.

Scale-type-specific requirement for the mosquito *A aegypti Spindle-F* homologue by regulating microtubule organization

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Abstract

Insect epithelial cells contain unique cellular extensions such as bristles, hairs, and scales. In contrast to bristle and hair, which are not divergent in their shape, scale morphology shows high diversity. In our attempt to characterize the role of the insect-specific gene, *Spindle-F* (*spn-F*), in mosquito development, we revealed a scale-type specific requirement for the mosquito *A. aegypti spn-F* homologue. Using CRISPR-Cas9, we generated *Ae–spn-F* mutants and found that *Ae–spn-F* is an essential gene, but we were able to recover a few adult escapers. These escapers could not fly nor move, and died after 3 to 4 days. We found that in *Ae–spn-F* mutants, only the tip part of the bristle was affected with bulbous with misoriented ribs. We also show that in *Ae–spn-F* mutants, only in falcate scales, which are curved with a sharp or narrowly rounded apex, and not in other scale types, the tip region is strongly affected. Our analysis also revealed that in contrast to *Drosophila spn-F*, which show strong defects in both the actin and microtubule (MT) network in the bristle, the *Ae–spn-F* gene is required only for MT organization in scales and bristles. In summary, our results reveal that *Ae–spn-F* is required for shaping tapered epithelial cellular extension structures, namely, the bristle and falcate scales by affecting MT organization.

Graphical Abstract

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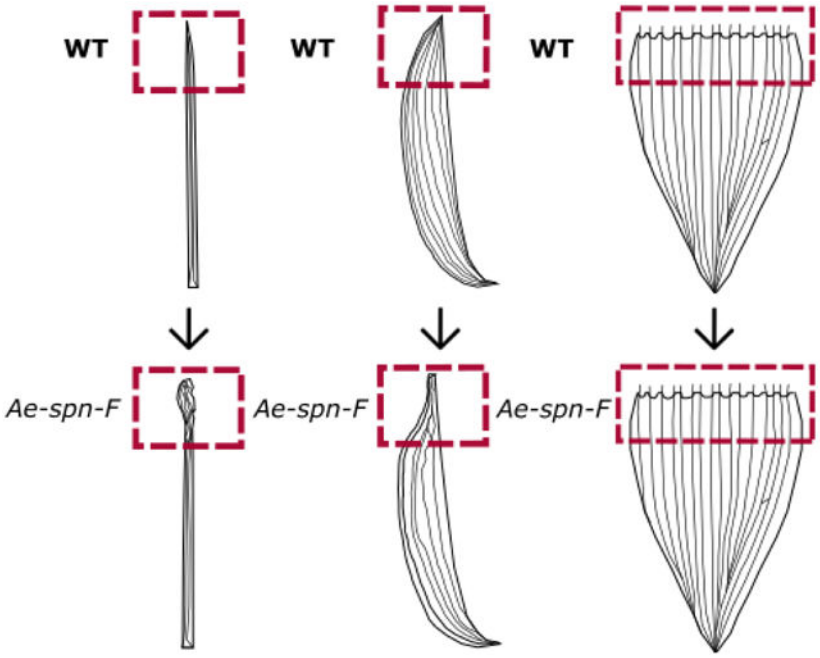
Author contribution

Investigation (performing of the experiments): SD, AB and ML. Investigation (data analysis): SD, UA and OSA. Writing/manuscript preparation (writing the initial draft): SD and UA. Writing/manuscript preparation (critical review, commentary, or revision): SD, UA and OSA. Funding acquisition: UA and OSA. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors have no conflict of interest to declare.



Keywords

Bristle; Microtubule; Mosquito; Scale; Spindle-F

Introduction

The insect cuticle contains cellular extensions; namely bristles, hairs, and scales(Guild et al., 2005). Most of our knowledge on molecular mechanisms that control these extension morphologies, mainly bristles, are from studies conducted in *Drosophila*. Cell cytoskeleton plays important role in bristle development, it was shown that in *Drosophila* bristle actin is required for cell elongation and Microtubules (MTs) are essential for maintaining the highly biased axial growth of the *Drosophila* bristle (Fei, He and Adler, 2002) and for mediating protein and membrane transport (Tilney et al., 2000a; Fei, He and Adler, 2002). The vast majority of genes that affect bristle development are actin-associated genes, which includes highly conserved genes such as *forked* (Petersen et al., 1994), *fascin* (Cant et al., 1994), *mical* (Hung et al., 2010), *twinfilin* (Wahlström et al., 2001) *profilin* (Giansanti et al., 1998), *capping protein* (Tilney and DeRosier, 2005), *cofilin* (Blair et al., 2006), *abp1* (Koch et al., 2012) and *arp2/3* complex (Koch et al., 2012). Among the insect specific actin associated protein is Javelin (Shapira et al., 2011). A second group of genes that affect bristle development are associated with MT organization, which includes mainly MT motor proteins Kinesin heavy chain (Brendza et al., 2000), Kinesin light chain (Melkov et al., 2015) and Dynein heavy chain 64C (Melkov et al., 2016). The third group includes genes that are part of the endosomal pathway, namely Rab11 (Nagaraj and Adler, 2012), Dusky-like (Nagaraj and Adler, 2012) and Rab35 (Zhang et al., 2009). The fourth group of genes is a novel complex protein, called Spindle-F (Spn-F) including, the insect-specific genes *spn-F* (Bitan, Guild and Abdu, 2010), *javelin-like* (Dubin-Bar et al., 2011) and the

Drosophila IKKε homologue- *ik2*, (Oshima et al., 2006). The current model by which Spn-F complex function suggests that, in the bristle, Spn-F and IKKε are transported to the distal bristle tip by cytoplasmic dynein, whereas Jvl is independently transported to the tip. At the distal tip, Jvl interacts with Spn-F and retains the IKKε–Spn–F complex. At the bristle tip, high accumulation of Jvl stabilizes MTs at the bristle tip where Spn-F and IKKe/IKKε accumulate to stimulate tip growth by activating endosomal trafficking (Otani et al., 2015; Otani et al., 2011, (Baskar et al., 2019)

In insects, it has been suggested that thoracic bristles, wing hairs, and scales are homologous structures that differ in their morphology1,2. Similarly to bristle, scales from the mill moth (*Ephestia kuenilla*), contains membrane-associated actin bundles, and cytoplasmic MTs (Overton, 1966). In butterfly, *Vanessa cardui*, actin bundles are required for initial scale elongation and to orient the scale parallel to the wing membrane (Dinwiddie et al., 2014). In mosquito, actin bundles are required for scale width formation (Djokic et al., 2020). Till that end the exact role of MTs in scale development is still unknown.

To understand the role of cytoskeleton in mosquito scale development, we decided to further analyze the role of the Spn-F complex, mainly Spn-F gene, on mosquito bristle and scale development. Specifically, *Spindle-F (spn-F*) was first identified as a maternal effect mutation that affects the dorsal–ventral polarity of the eggshell. Further studies revealed that in *spn-F* mutant, bristles are highly defected, being much shorter and wider as compared to WT. Our current knowledge on the function of spn-F complex suggested that Jvl is responsible for tethering Spn-F and IKKε at the bristle tip by serving as a molecular brake or as a scaffolding protein or by directly affecting MT organization at the bristle tip (Otani et al., 2011), (Otani et al., 2015), (Otani et al., 2016), (Dubin-Bar et al., 2008), (Bitan, Rosenbaum and Abdu, 2012), (Shapira et al., 2011), (Baskar et al., 2019)

Bioinformatic and molecular analyses reveal that *A. aegypti* AAEL014720 gene is homologous to the *Drosophila spn-F* gene, mentioned hereafter *Ae-spn-F*. We generated *A. aegypti spn-F* null mutants line using CRISPR/CAS9 and reveal that *A. aegypti spn-F* is mostly an essential gene. We were able to recover several escapers adults' mosquitos. Examination of bristle development in *Ae-spn-F* showed that bristles have the same length as Ubl-Cas9 bristles (used as control) and the only defect found in these bristles were their tip morphology. In *Ae-spn-F* mutant only one specific type of scale was affected. These scales have falcate shape, a sickle-shaped lamellar scale with a sharp or narrowly rounded tip. The other most common scale, which was not affected in *Ae-spn-F* mutant, have spatulate shaped, rounded and broad at the tip, and attenuated at the base. In both *Ae-spn-F* mutant bristle and scales, only MT but not actin organization was strongly affected. Thus, our study reveals that *Ae–spn-F* is required for shaping only tapered epithelial cellular extension structures, namely, the bristle and falcate scales by affecting MT organization.

Results

A. aegypti AAEL014720 gene is homologous to the *Drosophila spn-F* gene

To better understand the role of cytoskeleton in *A. aegypti* scale development, we chose to focus on identifying and analyzing the role of the *A. aegypti Spindle-F* (*Spn-F*) homolog

gene. In *Drosophila*, the *spn-F* (*Dm-spn-F*) gene has an important role in oocyte and bristle development, probably by affecting MT and actin organization (Abdu, Bar and Schüpbach, 2006); (Dubin-Bar et al., 2008); (Bitan, Guild and Abdu, 2010); (Amsalem et al., 2013). To identify this gene, we blasted the mosquito genome and identified AAEL014720 as a possible *spn-F* homolog. Our bioinformatics analysis revealed that the *A. aegypti* AAEL014720 gene shared 38.5% sequence identity and 54.7% similarity with the *Drosophila* Spn-F protein (Fig. 1A). We also noticed that whereas the *Drosophila* Spn-F protein has two coiled–coil domains, the *Ae*-Spn-F protein contained only one coiled–coil domain, (Fig. 1B). Also, as reported by (Lin et al., 2015), both *Dm-spn-F* and the *A. aegypti* AAEL014720 protein also contain the conserved Spn-F C-terminal domain (Fig. 1B). Taken together, our results suggest that the *A. aegypti* AAEL014720 gene is probably the homolog for *Dm-spn-F*, which will be referred to throughout the rest of the text as *Ae-spn-F*.

Genetics characterization of *A. aegypti spn-F* mutants

To generate an *Ae-spn-F* mutant line, sgRNA targeting exon 1(Fig. 2A) was injected into eggs of a homozygous mosquito line that expressed Cas9 under the control of a ubiquitin L40 (AAEL006511) promoter (Li et al., 2017). Injection of sgRNA into 200 embryos yielded 49 Generation 0 (G0) mosquitoes. All of these were crossed with WT mosquitoes of the opposite sex. Next, eggs from one of the G1 generation lines were hatched, and all 39 offspring were PCR-screened for detection of Cas9-generated mutations. Among the 39 screened adults, 17 (43.6%) had one visible PCR product of the expected size of the WT allele (Fig. 2B), and 22 (56.4) individuals had two PCR products [one with the expected size of the WT allele and a second smaller PCR product indicating a deletion (Fig. 2B)]. DNA sequencing of the smaller band revealed a 47-bp deletion (Fig. 2C) at the sgRNA site in the *Ae–spn-F* gene, resulting in a frameshift, followed by 39 unrelated aa genotypes, and then a stop codon (Fig. 2D). Next, 11 males from the 22 G1 heterozygous mosquitoes were grouped and crossed with WT female mosquitoes. To generate homozygous *Ae–spn-F* mutant individuals, G2 heterozygous (*Ae–spn-F/+)* male and female were mated, and homozygous (*Ae–spn-F/ Ae–spn-F)* individuals were identified based on PCR-analysis. We found that the *Ae–spn-F* gene was mostly essential for mosquito development, as most of the PCR-identified homozygous *Ae–spn-F* mutants (Fig. 2B) died as pharate adults (i.e. still in pupal casing). Out of the 92 adult's mosquitos that emerged, 26 (28%) were WT, 47 (51%) were heterozygous and 11 (about 12%) were homozygous. From the 11 homozygous mosquitos, 4 were alive (0.4%, escapers), which were not able to fly or move and died after 3 to 4 days. Our efforts to feed *Ae–spn-F* mutant female with blood were unsuccessful preventing us from analyzing defect in ovarian development post-blood feeding.

A. Aegypti spn-F gene is required for shaping the tip of the mosquito bristle and scales

Given that in *Drosophila*, *spn-F* is essential for bristle development (Abdu, Bar and Schüpbach, 2006), we used scanning electron microscopy (SEM) to examine the nature of the defects in both bristles and scales in *Ae–spn-F* adult escaper mutant lines. Analyzing bristle morphology in Ubl-Cas9 (as control) (Fig. 3A-C) and in *Ae–spn-F* escapers mutants (Fig. D-F) revealed that the mutant bristles were similar in length (330.71±8.50 μm) to the Ubl-Cas9 bristles (309.97±9.86 μm). However, closer examination of the bristle tip region showed that compared to the Ubl-Cas9, which had tapered tips (Fig. 3B), the mutant

bristle tips had a bulbous shape with misoriented ridges and valleys (Fig. 3E). The effect on bristle development was restricted to the tip region, as the middle part of the bristle showed WT-like organization (Fig. 3, compare C to F).

Next, we analyzed whether *Ae–spn-F* has a role in scale development. We analyzed scale morphology in the *Ae–spn-F* mutants and found that in scales from the thorax (Fig. 4A as compared to 4B) and wings (Fig. Fig. 4C as compared to 4D), only the tip region of the scales was strongly affected. On the other hand, the morphology of scales from the legs (Fig. 4E as compared to 4F), scutellum (Fig. 4G as compared to 4H), and abdomen (Fig. 4I as compared to 4J) were not affected. To quantify the defects in the *Ae–spn-F* scales, we decided to measure the length and width of the scale tips on the thorax (Fig. 4K-L). First, we found that the length of the thorax scales from the mutants (60.67±1.73 μm) was like Ubl-Cas9 (67.20±1.35 μm). To measure the width of the scales in the same region between the Ubl-Cas9 and *Ae–spn-F*, we measured the width of 1/10 of the upper part (Fig. K-L) of each of the scales. We found that the width of the scales from the *Ae–spn-F* (2.02±0.14 μm) was significantly ($t = 8.7573$, $df = 10$, $p<0.0001$) (Fig. L) narrower compared to the scale width from the Ubl-Cas9 (6.56±0.40 μm).

MT but not actin bundles are affected in both bristles and scales from *Ae–spn-F* mutants

Since both actin (Tilney, Tilney and Guild, 1995), (Tilney et al., 1996), (Tilney et al., 1998), (Tilney et al., 2000b), (Guild et al., 2003), (Guild et al., 2005) and MTs (Tilney et al., 2000a), (Fei, He and Adler, 2002), (Bitan et al., 2010), (Bitan, Rosenbaum and Abdu, 2012) are required for bristle and scale (Dinwiddie et al., 2014), (Djokic et al., 2020) development, we used confocal microscopy to visualize phalloidin staining for actin (Fig. 5A-D) and antibody staining for MTs (Fig. 5E-H). In contrast to Ubl-Cas9 bristles (Fig. 5A), in the *Ae–spn-F* pupa, phalloidin staining revealed that the tip of the bristles had a bulbous shape, but no obvious abnormality in actin localization was detected (Fig. 5A as compared to 5B). To analyze the cytoskeleton organization in scales, we focused on scales from the thorax, which were affected in *Ae–spn-F* pupa (Fig. 4B). In scales, no obvious defects in actin organization were evident in the *Ae–spn-F* (Fig. 5D) as compared to the Ubl-Cas9 (Fig. 5C). On the other hand, closer examination of MT organization in both bristles and scales revealed, that in both cells in Ubl-Cas9 mosquitos bristles (Fig. 5E) or scales (Fig. 5G), MTs are distributed equally throughout the shaft, whereas both in bristles (Fig. 5F) and scales (Fig. 5H) from *Ae–spn-F* mutants, MTs were abnormally distributed throughout the cell shafts, as evident from their high aggregate accumulation throughout the shaft.

Discussion

Identification of the *A. aegypti* AAEL014720 gene as *Drosophila spn-F* gene homolog

Bioinformatics analysis revealed that the *A. aegypti* AAEL014720 gene is the predicted *Drosophila spn-F* gene homolog. This gene shares 38.5% sequence identity and 54.7% similarity with the *Drosophila* Spn-F protein. It was also found that it contains the conserved Spn-F C-terminal domain (SCD) (Lin et al., 2015). On the other hand, *Drosophila spn-F* contained two coil–coiled domains, the AAEL014720 gene contained only one. Previously, we and others found that Dm-Spn-F physically interacts with Ik2, a serine/threonine kinase

and a *Drosophila* homolog of vertebrate IKKε. It was demonstrated that Ik2 phosphorylates Spn-F (Dubin-Bar et al., 2008); (Lin et al., 2015); (Otani et al., 2015), and it was shown that eight serine residues (S53, S85, S172, S202, S264, S270, S325, and S349) of Spn-F are phosphorylated by Ik2 kinase (Lin et al., 2015). Comparing the conservation of these eight residues in the *A. aegypti* AAEL014720 protein revealed that only S172, S325, and S349 are conserved, and that S264 changes to threonine. Thus, at least half of amino-acid residues that are phosphorylated by Ik2 are conserved in *Ae–spn-F.* To support, our claim that the *A. aegypti* AAEL014720 gene is the *Drosophila spn-F* gene homolog, we generated an *A. aegypti* AAEL014720 mutant line using CRISPER/Cas9 and showed that this is required both for mosquito viability and for bristle and scale development, as expected from the role of the *Drosophila* Spn-F phenotypes (Abdu, Bar and Schüpbach, 2006). Based on the above observations, we conclude that the *A. aegypti* AAEL014720 gene is the *Drosophila spn-F* gene homolog.

Ae–spn-F affects only MT organization but not both MTs and actin as the *Drosophila Spn-F* gene

Although we conclude that *A. aegypti* AAEL014720 is the *Drosophila spn-F* gene homolog, we did find some differences between the molecular function of these two homologous genes concerning bristle phenotypes. First, in *Drosophila spn-F* mutants, bristles are shorter and thicker, and the direction of bristle growth at some points along the bristle shaft is altered (Bitan, Guild and Abdu, 2010). On the other hand, in *Ae–spn-F*, the length and width of the bristle are not affected, and the only phenotype exhibits defects in tip morphology. Secondly, in *Drosophila spn-F* mutants, the actin bundles are not restricted to the shaft periphery, and the actin modules are poorly oriented, resulting in adult actin ridges that run in different directions. However, in the *Ae–spn-F* mutant, no obvious defects in bristle actin organization were found.

In contrast to the difference in the function of these two homologous genes in bristle development, as described above, both *Drosophila-spn-F* and *Ae–spn-F* have the same role in bristle MT organization. In *Drosophila spn-F* (Otani et al., 2015) and in *Ae–spn-F*, bristle MTs were extremely disorganized, in which MTs often appeared as aggregates found at various locations along the bristle shaft. More interestingly, in scales from the *Ae–spn-F* mutant, MTs, but not actin, were severely affected, showing aggregates in the scale shaft. Thus, our results suggest that whereas *Drosophila spn-F* is required for both bristle actin and MT function, *Ae–spn-F* is required only for bristle and scale MT organization. Our previous analysis failed to distinguish what the primary function of *Drosophila -spn-F* is; is it required directly for bristle actin organization and then indirectly if it affects bristle MT organization or vice versa, or maybe functions in both cytoskeleton networks. Our results that *Ae–spn-F* is required only for MT organization suggest that at least in the mosquito the gene is required directly for bristle and scale MT organization.

Differential requirement of *Ae–spn-F* in scale development

Beside bristles, the body of the mosquito is covered with another homologous bristle structure, namely, scales (Wu, Kong and Wu, 2007). Scales also contain actin bundles that dictate their cellular morphology and an MT network whose function in shaping

scale morphology is unknown. The entire body of the mosquito is covered with two morphologically different types of scales. The first type is spatulate shaped—rounded and broad at the tip, and attenuated at the base. These scales can be found on the abdomen, leg, and scutellum. The second most common scale has a falcate shape—a sickle-shaped lamellar scale in which the squame is narrow and curved with a sharp or narrowly rounded apex. These scales can be found on the margins of the wing and on most parts of the thorax. Our results demonstrate that *Ae–spn-F* is required for shaping the morphology of only the falcate-shaped scales, specifically at the narrow tip. Given that in the bristle, *Ae–spn-F* is also required in shaping the tapered-narrower tip, suggests that *Ae–spn-F* has a role in tapering the tip of certain insect cellular epithelial extensions such as bristles and falcate scales.

Experimental procedures

Aedes aegypti mosquito strains and rearing

Injections into embryos were performed by using *A. aegypti* mosquitoes expressing Cas9 protein under the control of the ubiquitin L40 (Ubl-Cas9) promoter (Li et al., 2017). *Aedes Aegypti* mosquito colony wild type (WT) was another line that were used in experiments. In all of our experiments Ubl-Cas9 were used as control. All mosquitoes in this paper were maintained in a growth chamber set at 27 °C with 60%-75% relative humidity on a 12 h light/12 h dark cycle. Larvae were reared at 27 °C and 75% humidity in water and fed with a mixture of Tetramin fish flakes and yeast (1:1 ratio) until pupation. Adults were maintained with 10% sucrose. Female mosquitoes were fed on mouse blood using a Hemotek feeder (PS-6 System, Discovery Workshops, Accrington, UK). Blood (3.0 mL) was transferred into the Hemotek blood reservoir unit at 37 °C.

Single guide RNAs (sgRNA) synthesis

Oligonucleotide used in this study was sgRNA 5′ GGAGATCACAAACGAAAGGA 3′ targeting exon 1 in the *Ae-spn-F* gene. The primers for sgRNA design were annealed using HS Taq Mix Red DNA Polymerase. sgRNA templates were transcribed using T7 polymerase from a TranscriptAid T7 High Yield Transcription Kit. RNA transcripts were purified by a Phenol/Chloroform RNA extraction.

Microinjections of embryos

The *A. aegypti* embryo microinjections were similar to those previously described (Jasinskiene, Juhn and James, 2007). Four days after blood feeding, ten mated gravid females were transferred into plastic tubes with water- filled cotton covered above with Whatman paper and kept in the dark for oviposition. After 30 min, eggs were collected and aligned on a piece of Whatman filter paper. Eggs were covered with a 1:1 mix of Halocarbon 700 oil:Halocarbon 27 oil to prevent desiccation. Glass needles were pulled using a Narishinge model PP-830 needle puller and were used with a Nikon eclipse injector. 0.2 nL sgRNA at 3537,62 ng/μL was injected into the embryos. All injected embryos were allowed to recover under insectary conditions for 5 days before hatching them into water.

Molecular analysis of mutant individuals

To ensure transmission of G0 mutation to the next generation, G0 adults were screened for bristle and scale defects. Individuals with phenotype were crossed with WT mosquitoes and kept in cups of one female and three male and opposite. All G1 progeny were screened using legs as a source for genomic DNA with NaOH Extraction DNA as an extraction tool. Genomic DNA was used as a template for PCR with the following primers: Forward 5′ CCGGAAAAAGATAACTGTTC 3′ and reverse 5′ GAAAGCTACAGGCAACGATA 3′. PCR products were separated in 3% agarose gel electrophoresis, eluted from an excised band of the gel, and directly sequenced.

Pupa phalloidin and antibody staining

For examination of bristles and scales on confocal microscopy, 20-h old pupae were collected from controls (untreated pupae), or *Ae-spn-F* pupae (from a cross between heterozygous *Ae-spn-F* mosquitoes) and one part of the body was fixed in 4% paraformaldehyde in PBS overnight. Another part of the body of *Ae-spn-F* pupae was taken for DNA extraction and analyzed as described in molecular analysis of mutant individuals. For phalloidin staining, samples were washed three times with 0.3% Triton X-100 in PBS for 10 min each time, followed with 1% Triton X-100 in PBS for 1h and then incubated overnight with phalloidin. Then the samples were washed three times in 0.3% Triton X-100 in PBS. For antibody staining, samples were washed three times with 0.3% Triton X-100 in PBS for 10 min each time, followed with 1% Triton X-100 in PBS for 1h and then the thoraces were blocked in 0.1% Triton X-100 containing 4% bovine serum albumin for 1 h. The samples were then incubated overnight with a primary antibody in the blocking solution at 4 °C, washed three times in 0.3% Triton X-100 in PBS, and incubated with secondary antibodies in blocking solution for 2 h at room temperature or at 4 °C overnight in the dark. After incubation, samples were washed three times with 0.3% Triton X-100 in PBS for 10 min each time. For confocal observation, both phalloidin and antibody-stained samples were placed on a slide and mounted in 50% glycerol. A coverslip was placed on the sample, and the preparation was sealed with nail polish. The slides were examined with an Olympus FV1000 laser-scanning confocal microscope. Mouse anti-α-tubulin (1:250) (Sigma) primary antibodies were used. Goat anti-mouse Cy2 secondary antibody were used at a dilution of 1:100. For actin staining, we used 568-conjugated phalloidin (1:250) (Molecular Probes).

Scanning electron microscopy (SEM)

Samples were fixed and dehydrated by immersion in increasing concentrations of ethanol (30%, 50%, 75%, and twice in 100%; 10 min each). The samples were then completely dehydrated using increasing concentrations of hexamethyldisilazane (HMDS) in ethanol (50%, 75%, and twice in 100%; 2 h each). The samples were air dried overnight, placed on stubs, and coated with gold. The specimens were examined with a scanning electron microscope (SEM; JEOL model JSM-5610LV). Length measurements of adult bristles were performed using Image J (https://rsb.info.nih.gov/ij/) (version 1.40j) software.

Acknowledgements

We thank Einat Nativ-Roth for her help with the scanning electron microscope. This work was supported in part by funding from NIH awards (R01AI151004 and DP2AI152071) to O.S.A.

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- Mosquito spindle-F (*Ae-spn-F*) mutants were generated using CRISPR/CAS9 system.
- *Ae–spn-F* is required for shaping tapered epithelial cellular extension structures, namely, the bristle and falcate scales.
- *Ae-spn-F* regulates tapered epithelial cellular extension by microtubule organization

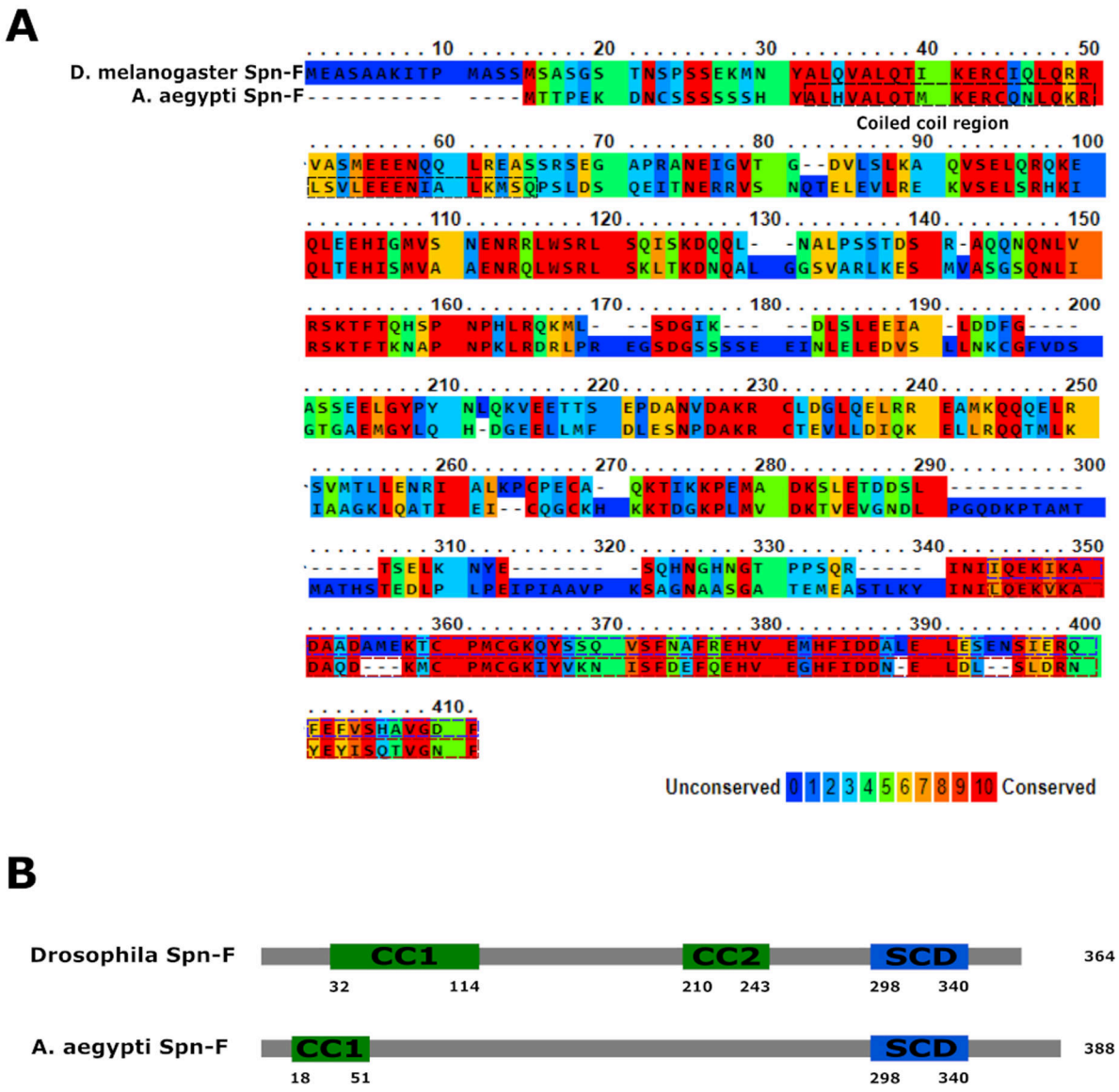

Figure 1. Identifying the *A. aegypti* AAEL014720 gene as a *Drosophila* Spn-F putative homolog.

A) Alignment of Spn-F *Drosophila* protein with *A. aegypti* AAEL014720 revealed 38.5% sequence identity and 54.7% similarity. Coiled–coil domain of *A. aegypti* (black box). Conserved Spn-F C-terminal domain in *Drosophila* (blue box) and *A. aegypti* (red box). **B)** Bioinformatics information revealed that whereas *Drosophila* Spn-F protein contained two coiled–coil domains (green boxes), *Ae*-Spn-F protein contained only one coiled–coil domain (green box). The conserved Spn-F C-terminal domain in both *Drosophila* and *A. aegypti* (blue box).

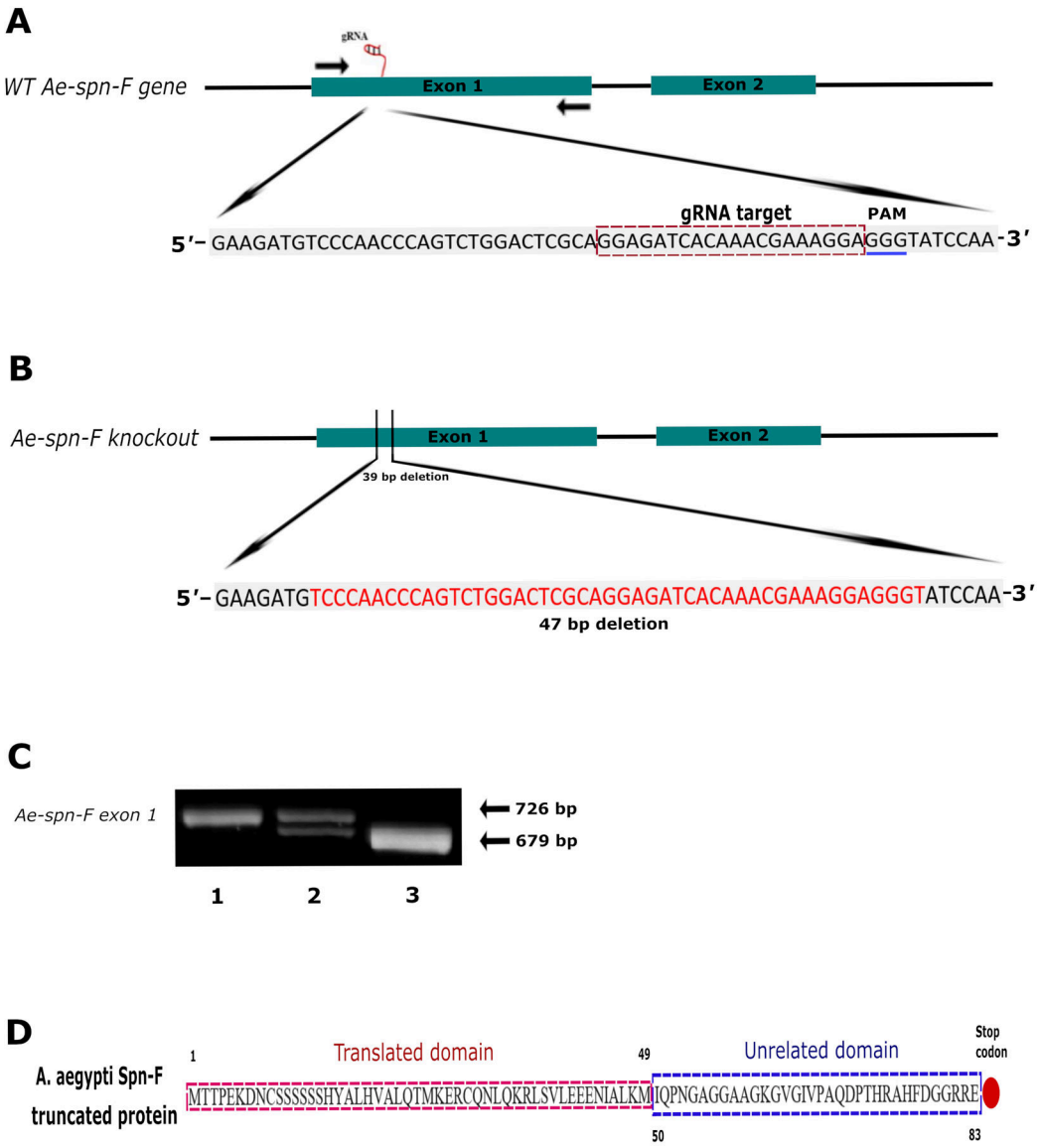

Figure 2. Generation of *Ae–spn-F* mutants using the CRISPR/CAS9 system.

A) Genomic organization of the *Ae–spn-F* gene. The green boxes are exons, the black lines represent 5', 3' UTRs and introns. sgRNA on exon 1 is written; primers that were used to characterize (PCR results in C) *Ae–spn-F mutants* are marked in black. sgRNA was injected into *A. aegypti* transgenic mosquito embryo ubiquity expressing CAS9 protein under *ubiquitin L40* (AAEL006511) promoter were used (Li et al., 2017). DNA sequence of genomic DNA from wild-type (WT) around the sgRNA site (the upper line at the purple end of the PAM site is marked with a box). **B)** PCR analysis of WT and mutant mosquito lines. (1) PCR on WT mosquitoes reveals a PCR product of 726 nt. (2) PCR on G1 putative mutant heterozygous lines reveals two PCR products, one the same size as in WT, and the second smaller in size, about 679 nt. (3) PCR on a homozygous G3 *Ae–spn-F* line showing only one band, which represents the deletion of 679 bp. **C)** DNA sequence of genomic DNA from the *spn-F A.aegypti* mutants. Deletion of 47 bp is marked in red. **D)** Result of a

47-bp deletion is truncated Spn-F protein, which appears to be 83 aa long. Truncated protein contains translated 49 aa (marked with dotted red lines), followed by a frame shift following 39 unrelated amino acids (marked with blue dotted lines) and a stop codon (red circle).

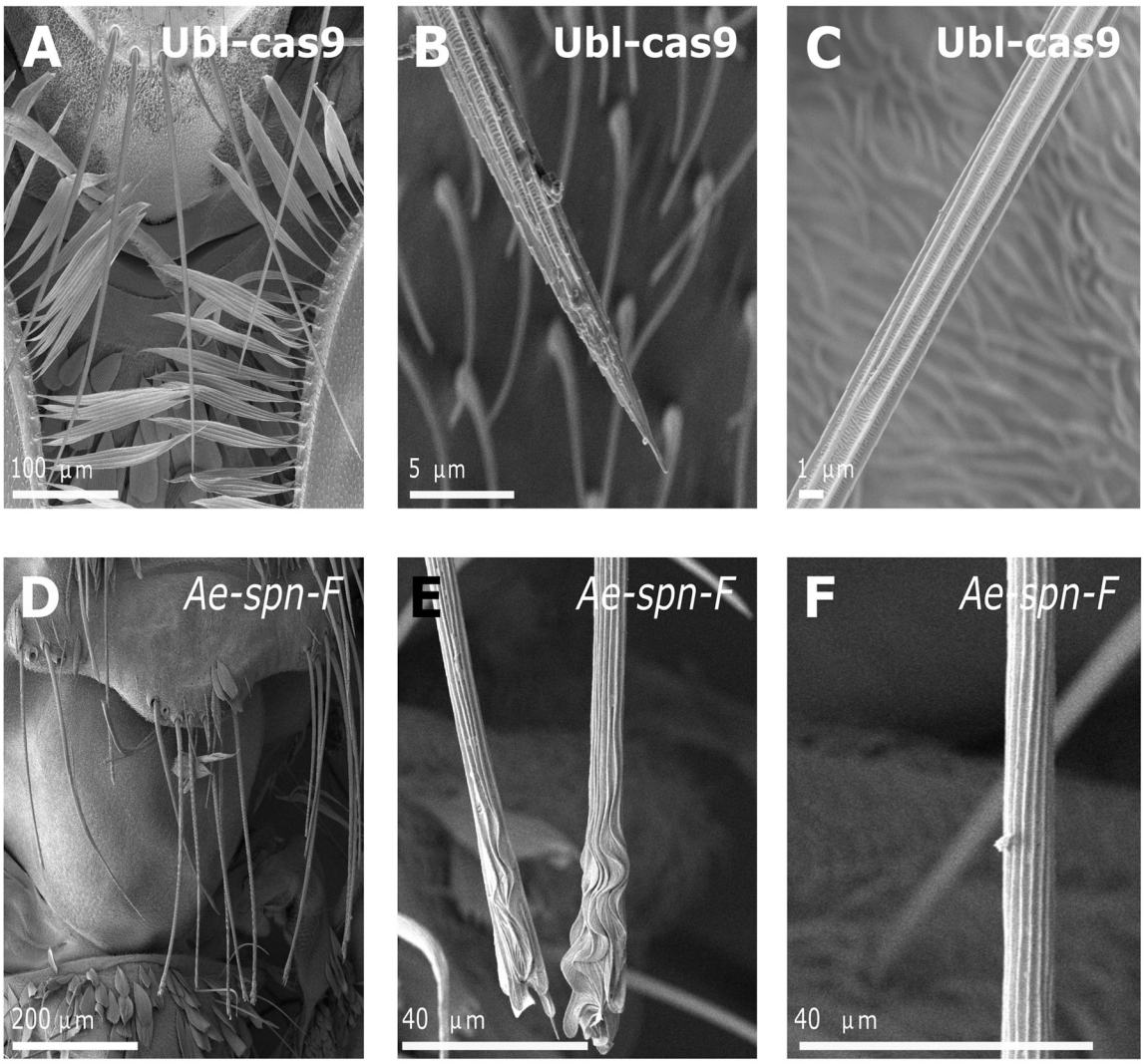

Figure 3. Scanning electron microscopy (SEM) images of bristles from *Ae–spn-F* knockout mosquito by CAS9/CRISPR.

A) Scutellum from wild-type (WT) adult mosquito. **B)** Closer examination of WT mosquito bristle tip showing tapering toward the tip with parallel positioned ridges and valleys. **C)** Middle part of the WT bristle. **D)** Scutellum from *Ae–spn-F* knockout mutant mosquito. **E)** Closer examination of *Ae–spn-F* knockout mutant mosquito bristle showing defects only on tip of the bristles. *Ae–spn-F* knockout mutants reveal that instead of tapering toward the bristle tip on WT **(B)**, *Ae–spn-F* mutant bristles become bulbous at the tip with misoriented ridges and valleys, thus, losing its sharp tip phenotype. **F)** Middle part of *Ae–spn-F* bristle shows WT-like organization.

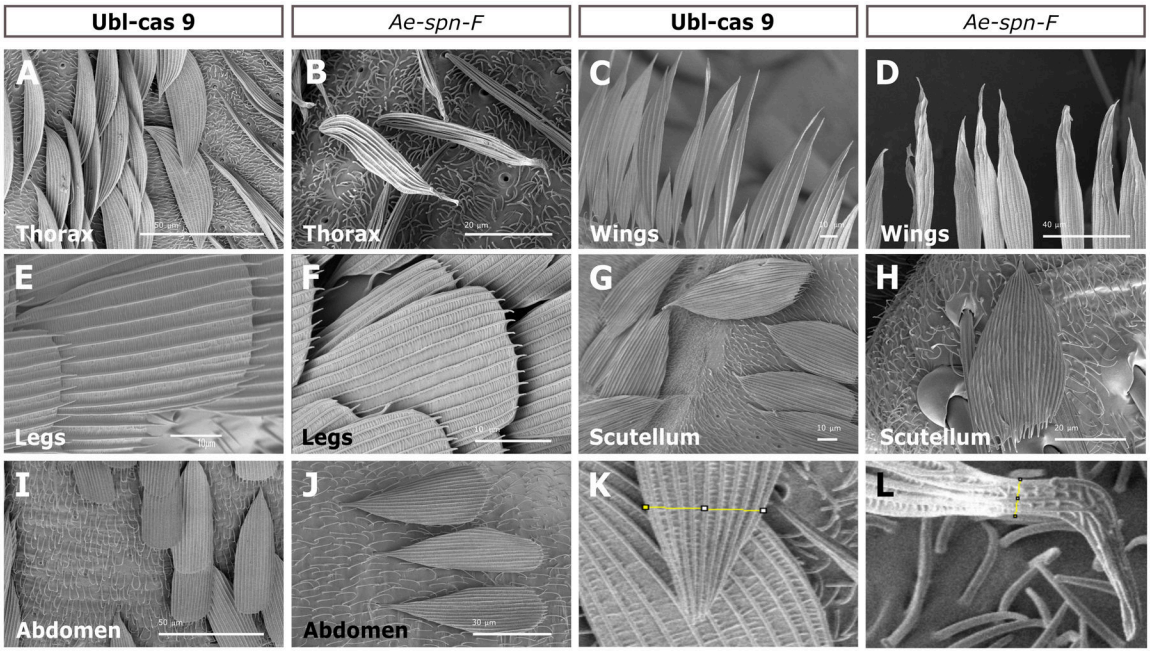

Figure 4. Scanning electron microscopy images of scales from different body regions of the *Ae–spn-F* knockout mosquito by CAS9/CRISPR and WT with a focus on scales.

WT scales on the thorax **A)**, wing **C)**, leg **E)**, scutellum **G),** and abdomen **I)** of an adult mosquito. *Ae–spn-F* scales on the thorax **B)**, wing **D)**, leg **F),** scutellum **H),** and abdomen **J)** of an adult mosquito. Point of width measurement (yellow line) in a WT thorax scale **K)** and an *Ae–spn-F* thorax scale **L)**. Closer examination of *Ae–spn-F* knockout mutant both on a thorax scale **B)** and a wing scale **D)** showing misoriented ridges and valleys on the tip. No morphology defects in scales on the leg (compare E to F), scutellum (compare G to H), or abdomen (compare I to J) from *Ae–spn-F* compared to WT were found.

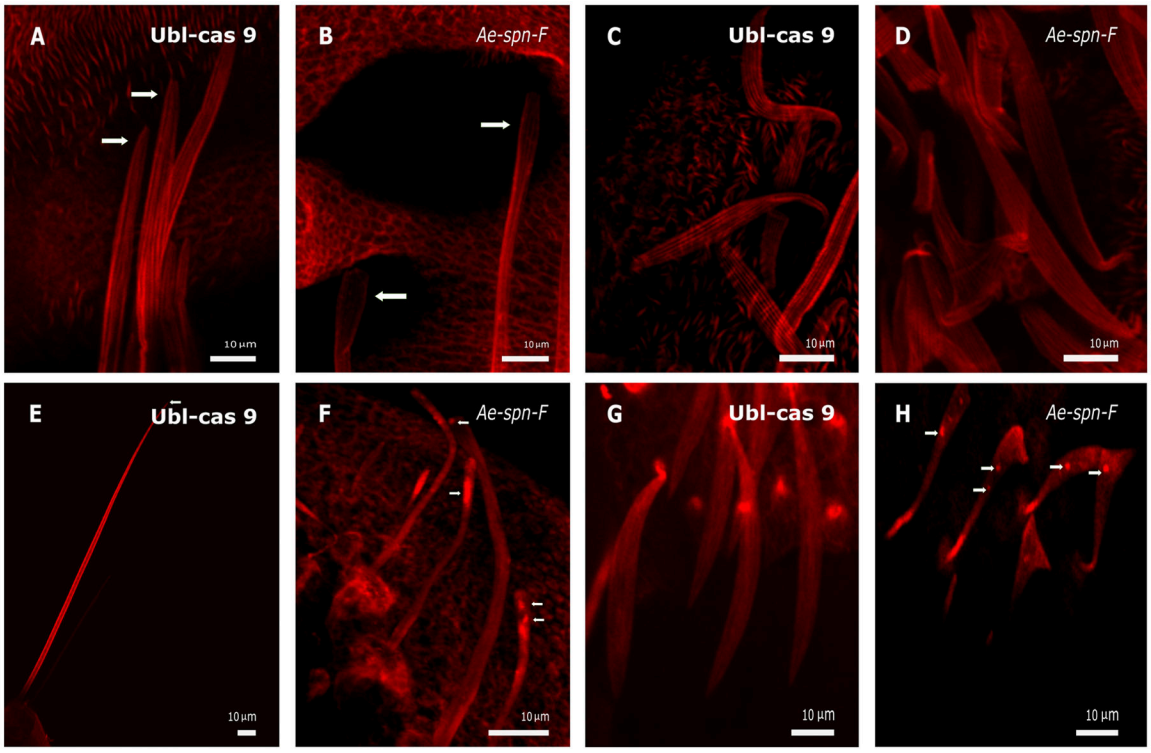

Figure 5. Confocal microscopy images of actin and tubulin staining from WT and *Ae–spn-F* knockout mosquito bristles and scales.

Actin staining of WT mosquito bristle **A)** showed parallel actin bundles along the entire bristle with tapered tip (arrows). **B)** Actin staining of WT mosquito bristle mutant bristles revealed WT-like organization of actin bundles; however, the tip of the bristles has a bulbous shape (arrow). Actin staining of WT **C**) or *Ae–spn-F* **D)** mosquito thorax scales shows similar actin distribution. Tubulin staining of a bristle from a WT **E)** adult mosquito shows that MTs are evenly distributed throughout the bristle shaft. The tapered tip of the bristle is marked with an arrow **E)**. **F)** Tubulin staining of *Ae–spn-F* mutants reveals an abnormal accumulation of MT clumps at the tip region (arrows). Tubulin staining of scale from a WT **G)** adult mosquito shows that MTs are evenly distributed throughout the shaft. **H)** Tubulin staining of *Ae–spn-F* mutants reveal an abnormal accumulation of MT clumps at the scale shaft (arrows).