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The extracellular matrix protein Artichoke is required for integrity of ciliated mechanosensory and chemosensory organs in *Drosophila* embryos

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ABSTRACT

Sensory cilia are often encapsulated by an extracellular matrix (ECM). In *Caenorhabditis elegans*, *Drosophila melanogaster*, and vertebrates, this ECM is thought to be directly involved in ciliary mechanosensing by coupling external forces to the ciliary membrane. *Drosophila* mechano- and chemosensory cilia are both associated with an ECM, indicating that the ECM may have additional roles that go beyond mechanosensory cilium function. Here, we identify Artichoke (ATK), an evolutionary conserved leucine-rich repeat ECM protein that is required for normal morphogenesis and function of ciliated sensilla in *Drosophila*. *atk* is transiently expressed in accessory cells in all ciliated sensory organs during their late embryonic development. Antibody stainings show ATK protein in the ECM that surrounds sensory cilia. Loss of ATK protein in *atk* null mutants leads to cilium deformation and disorientation in chordotonal organs, apparently without uncoupling the cilia from the ECM, and consequently to locomotion defects. Moreover, impaired chemotaxis in *atk* mutant larvae suggests that, based on ATK protein localization, the ECM is also crucial for the correct assembly of chemosensory receptors. In addition to defining a novel ECM component, our findings show the importance of ECM integrity for the proper morphogenesis of ciliated organs in different sensory modalities.

INTRODUCTION

Cilia are microtubule-based membrane projections with a microtubule-based cytoskeleton, or axoneme, which consists of nine circularly arranged microtubule doublets. Cilia have been traditionally classified into motile or primary ones on the basis of an additional central microtubule pair that can be present (motile cilia) or not (primary cilia) (Gerdes *et al.* 2009). Recent data suggest nonetheless that it is the presence of outer and inner dynein arms what determines cilia motility. In invertebrates, cilia are restricted to certain sensory neurons that innervate specialized chemo- and mechanosensory organs, and to sperm cells (Dubruille *et al.* 2002; Keil 2012).

Cilia can protrude freely into the extracellular space or be associated with an extracellular matrix (ECM) (McGlashan *et al.* 2006). This ECM seems especially important in the context of mechanotransduction. Given the exquisite speed of mechanotransduction, it is generally assumed that mechano-electrical transduction channels are directly gated by mechanical stimuli and detect the deflection of an external structure relative to an internal cellular structure (Gillespie and Walker 2001). The ECM acts as a linker between the external structure and the channels and is therefore an essential component of the system. Consistent with this hypothesis, different *mec* genes that were isolated in genetic screens in *C.elegans* for mechanosensitive mutants encode ECM proteins (Chalfie and Sulston 1981; Chalfie and Au 1989). Although the nature of the interaction between the ECM and the transduction channel is not fully understood, further studies in *C.elegans* have shown that the ECM proteins MEC-1 and MEC-5 are necessary for the localization of the mechanosensory degenerin channel complex and for touch sensitivity (Emtage *et al.* 2004).

In *Drosophila*, this sensory ECM has been described for all ciliated mechanosensory organs and it is specifically known as the dendritic cap. Ciliated mechanosensory organs are type I

sensory organs, which also include chemosensory organs. Type I sensory organs are multicellular and characteristically innervated by ciliated sensory neurons. Unlike type I organs, type II organs are unicellular, consisting of single multidendritic neurons whose dendrites lack cilia. In *Drosophila* embryos, mechanosensory type I organs can be further subdivided into two types: chordotonal (ch) organs that are attached internally to the cuticle and lack external structures, and external sensory (es) organs whose stimulus detecting structures protrude from the cuticle (reviewed in Kernan 2007). Both ch and es organs are innervated by a ciliated sensory neuron that is enveloped by accessory cells. They possess a dendritic cap at the cilium tip that is secreted by the accessory cells. Its molecular composition and how it is assembled is largely unknown: the only protein that has been reported to localize to the dendritic cap is the zona-pellucida (ZP)-domain protein NOMPA, which presumably serves as a scaffold to which other proteins bind (Chung *et al.* 2001). *nompA* mutants display disorganized dendritic caps, abolishing ch and es neuron function (Chung *et al*. 2001; Göpfert and Robert 2003). NOMPA also localizes to the dendritic tips of the ciliated neurons innervating *Drosophila* chemosensory organs (Chung *et al.* 2001), but the role of the supporting ECM has not yet been reported for these organs.

Since ECMs are common to all ciliated sensory organs, and are known to be essential at least in mechanosensation, it seems crucial to understand how the connections within the ECM scaffold occur. Adhesion molecules like leucine-rich repeat (LRR) proteins are likely to play an important role in this process. LRR are protein-protein interaction motifs of 20-30 amino acids enriched in leucines (Dolan *et al.* 2007). In *C. elegans*, the LRR proteins LET-4 and EGG-6 and the secreted LRR protein SYM-1 are required for the organization of the apical ECM in the excretory duct and pore (Mancuso *et al.* 2012). In *Drosophila*, the LRR secreted protein Convoluted (Conv) is necessary for apical matrix organization during tracheal tube

morphogenesis (Swanson *et al.* 2009). However, whether LRR proteins play also a role in the organization of the ECM supporting sensory organs is unknown.

Here we report the identification of Artichoke (ATK, CG5195), a novel *Drosophila* secreted LRR protein that localizes to the supporting ECM of all ciliated sensory organs during the late embryonic stages of their morphogenesis. *atk* mutants generated by mobilization of the P{EPgy2} element $Uhg8^{EY07139}$ show defects in the stretching of mechanosensory cilia in the lateral pentaloscolopidial (lch5) organ, the most prominent embryonic ch organ, and are locomotion impaired. These anatomical and behavioral phenotypes are rescued if the P{EPgy2} element $Uhg8^{EY07139}$ is removed. Co-localization of ATK with NOMPA in embryonic chemosensory organs and mutant defects in chemotaxis document that ECM components are also required for chemosensation.

MATERIALS AND METHODS

Fly strains: w^{1118} and the P{EPgy2} element line $Uhg8^{EY07139}$ were obtained from Bloomington Stock Centre. ATK subcellular localization was analyzed using the line *GFP-nompA* (Chung *et al*. 2001) (kindly provided by D. F. Eberl, University of Iowa, Iowa City, IA, 52242).

Mutagenesis: Hypomorhic alleles atk^{8} and atk^{23} were generated by imprecise excision of the P{EPgy2} insertion $Uhg8^{EY07139}$. The null allele atk^{33} was generated by P-element induced male recombination (Preston and Engels 1996a; Preston *et al.* 1996b). PCR and sequencing to detect genomic deletions were performed using the following primers: 5'-CCTGCCTTCTTGATGACAACTT-3´ (forward), 5'-GATTCCTCCGGCTGAAGAG-3' (reverse). atk^{33} was sequenced from the inverse PCR product of the ligation of whole genomic

DNA after digestion with *Hpa*II, using the primers: 5'-CCTTTCACTCGCACTTATTG-3'(forward) and 5'-GTGAGACAGCGATATGATTGT-3' (reverse).

In situ hybridization: Whole-mount in situ hybridization was performed using digoxigenin RNA labeled probes (Tautz and Pfeile 1989). A 2.5 kb *Eco*RI *atk* antisense RNA probe was synthesized using the cDNA clone RE27764. Genotypes examined were w^{1118}, $Uhg8^{EY07139}$, atk^{8}, atk^{23}, atk^{33} and $Uhg8^{EY07139rv}$. Staining was detected using NBT/BCIP reaction (Roche, Basel, Switzerland).

Antibody generation: A fusion protein containing amino acids 1403-1523 of ATK was generated using genomic DNA as a template and the primer pair: 5'-GCGAATTCAGAGAATCTCCTTGTGCAATCG-3' (forward) and 5'-ATGGATCCCCACCAGCTTCTCCTCCACT-3' (reverse), containing *Eco*RI and *Bam*HI restriction sites respectively (underlined sequence). The digested fragment was cloned in the *glutathione-S-transferase* (*GST*) gene fusion vector pGEX-2T (Promega, Madison, WI, USA) and transformed in *E.coli* BL21 DE3. Selected clones were verified by sequencing.

After induction, the GST-$ATK_{1403-1523}$ protein was purified using the Profinia Protein Purification System (BioRad, Hercules, CA, USA) and used for antibody generation in guinea pig following conventional procedures according to directives of the European Commission (2003/65/CE) and Spain (RD 1201/2005; BOE 252/34367-91, 2005) regarding the protection of animals used for experimental and other scientific purposes. Purified anti- ATK serum was used at 1:250.

Immunohistochemistry: Eggs were grown at 25° and collected at the stage required based on midgut morphology (Hartenstein and Campos-Ortega 1985). Staining of whole-mount embryos was performed using standard techniques. Combined detection of *atk* mRNA and marker proteins in *Drosophila* embryos was performed as described in (Patel 1994). The

following primary antibodies were used: neuronal marker mAb22C10 (anti-Futsch), mAb2B10 (anti-Cut), anti-Prospero (Vaessin *et al*. 1991), mAb8D12 (anti-Repo) (1:50, all from Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-α-Tub85E (Matthews *et al.* 1990) (1:10, kindly provided by Adi Salzberg, Israel Institute of Technology, Haifa, Israel), anti-Suppressor of Hairless (1:100, Santa Cruz Primary Antibodies, Dallas, Texas, USA), and mouse anti-GFP (1:100, Roche, Basel, Switzerland). Secondary antibodies used were Alexa 488 donkey anti-rabbit, Alexa 555 donkey anti-mouse and Alexa 555 goat anti-guinea pig. Additionally biotinylated anti-rabbit and goat anti-mouse were used for non-fluorescent stainings (all antibodies at 1:500 from Invitrogen, Carlsbad, CA, USA). Imaging was performed on a Zeiss LSM710 confocal microscope (Carl Zeiss, Jena, Germany). Z-series were projected to obtain cross-sectional views using ImageJ v1.42 (NIH) software.

Larval Crawling Analysis: Single wandering third instar larvae were placed on the center of a 135 mm Petri dish filled with 1% agarose. Larval movement was recorded over a period of 100 seconds in an environment 25ºC room. Wild type, $Uhg8^{EY07139}$, atk^{8}, atk^{23}, atk^{33} and $Uhg8^{EY07139rv}$ larvae were tested. Digital video movies were captured with Moticam 350 camera (Edmund Scientific, Tonawanda, NY, USA) and digitalized with Motic Images 2000 (MicroscopeWorld, Carlsbad, CA, USA) 1.2 at 15 frames per second (fps).

Dynamic Image Analysis: ImageJ v1.42 (NIH) software was used for processing of the digitalized images. High contrast digital movie frames were analyzed using the ImageJ plugin Mtrack2, which generates a coordinate matrix with the centroid positions of larvae over time. Variables were calculated using Excel (Microsoft, Redmond, WA, USA). Direction changes were counted when the difference between the two angles formed by three vectors binding four consecutive time points was bigger than 30º. Path length was calculated by adding all the cartesian distances between two centroid coordinates for consecutive frames.

Gustatory tests: A rectangular surface was divided in two halves filled with either 0.5 M sucrose in 1% agarose or 1% agarose (Heimbeck *et al*. 1999). To avoid diffusion, plates were poured immediately before testing. Thirty third-instar feeding larvae were placed on the edge between surfaces and were allowed to freely move. The distribution of larvae was counted after 15 min. A response index (RI) was calculated for each genotype ($RI= N_s - N_c/ N_s+ N_c$, where N_s and N_c are the numbers of larvae present on sucrose and control areas, respectively).

Statistical Analysis: The variable "artichoke-like" phenotype or its usual transformations did not follow a normal distribution. Hence, we used the Kruskal-Wallis test, followed by the proper post-hoc procedure to assess statistical differences (Conover 1999). Larval crawling data were normally distributed, while gustatory RIs were transformed into their arccosine that followed a normal distribution. Both sets of data were analyzed by a one-way ANOVA followed by planned multiple pairwise comparisons (Bonferroni correction). All statistical analyses were performed with SPSS13.

RESULTS

***atk* is specifically expressed in accessory cells of embryonic ciliated sensory organs:** To determine which cells express *atk* we performed RNA in situ hybridization to whole embryos. Positive hybridization signal was observed in the peripheral nervous system (PNS) in a pattern consistent with *atk* expression in type I embryonic sensory organs, including ch and es mechanosensory organs (Figure 1A) and chemosensory organs of the head and tail (Figure 1B). Judging from the hybridizations, *atk* transcription takes place at embryonic stages 15 to 17. Transcription commenced in the head and the terminal sensilla at stage 15 (image not shown). At early stage 16, transcription started in mechanosensory organs along the body

segments. Stage 16 embryos showed the strongest expression. At stage 17, expression vanished in mechanosensory organs along the body segments and weakened in the head and terminal sensilla (not shown). This temporal expression pattern suggests a role for *atk* in the late development of embryonic ciliated sensory organs that are undergoing the last developmental steps to become functional during larval stages.

Type I sensory organs are multicellular, consisting of ciliated sensory neurons and several accessory cells. To assess which of these cells express *atk*, we counterstained *atk* hybridization signals with different antibodies. Lack of co-localization of *atk* RNA with the neuronal marker mAb22C10 signaled that *atk* is not expressed in the sensory neurons but in associated accessory cells (Figure 1D,D',D''). It also showed that *atk* is not expressed in multidendritic type II sensory organs.

Ch organs contain five accessory cells: the scolopale cell, which enwraps the cilium and creates a receptor lymph cavity; the ligament cell that proximally suspends the organ (Bodmer *et al.* 1989); the cap cell, which connects the tip of the cilium to the cuticle via an extracellular cap (Hartenstein 1988; Carlson *et al.* 1997a; Yack 2004) and two epidermal attachment cells that, suspending the cap and the ligament cells respectively, seem to keep the organ under stretch (Brewster and Bodmer 1995; Inbal *et al.* 2004). Most accessory cells, including the ligament, the cap, and the two epidermal attachment cells, express α-Tubulin85E (α-Tub85E) (Matthews *et al*. 1990; Inbal *et al*. 2004). Double staining with *atk* RNA and anti-α-Tub85E yielded co-localization in one of these accessory cells that, judging from its position, is deemed to be the cap cell (Figure 1G,G'). This notion was corroborated when we counterstained with anti-Repo and anti-Prospero, which stain the ligament cell (Halter *et al*. 1995) and scolopale cell (Vaessin *et al*. 1991) respectively, but not the cap cell.

No co-localization was seen between *atk* RNA and these antibodies (data not shown), as expected for a cap cell expression of *atk*.

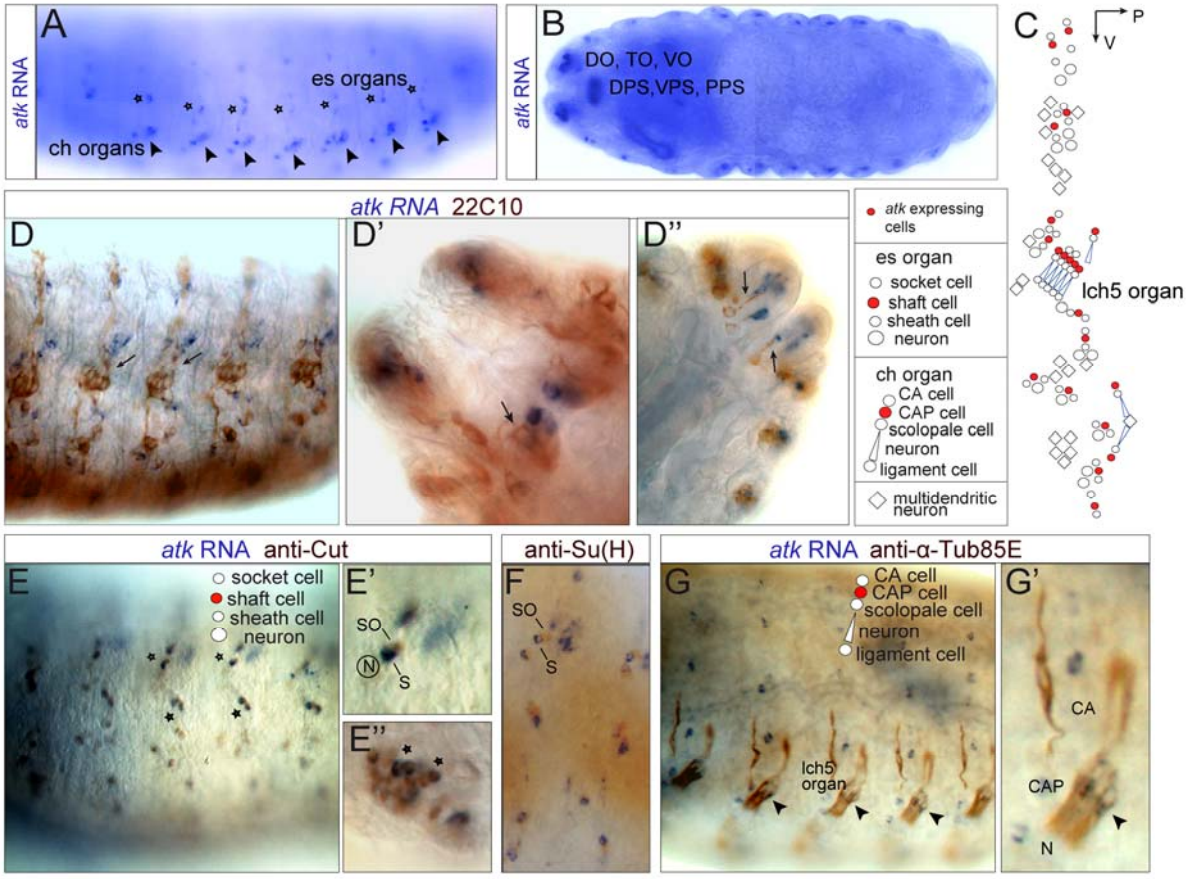

Figure 1. *atk* is expressed by an accessory cell in every ciliated sensory organ. (A-B) *atk* in situ hybridization in wild type stage 16 embryos. (A) Lateral view showing *atk* in situ signal in a pattern consistent with expression in ch organs (arrowheads) and es organs (stars). (B) Horizontal view showing *atk* expression in chemosensory organs: dorsal organ (DO), terminal organ (TO), ventral organ (VO), dorsal, ventral and posterior pharyngeal organs (DPS, VPS, PPS respectively). (C) Schematic diagram of mechanosensory organs per hemisegment showing *atk* expressing cells (red). (D-G) Embryos double-stained for *atk* in situ (blue signal) and different primary antibodies (brown signal). (D) *atk* is not expressed by ciliated neurons labeled by the neuronal marker mAb 22C10 (arrows, D-D''). (E-F) In es and chemosensory organs, *atk* is expressed by the shaft cell: it co-localizes with anti-Cut signal (stars, E-E''), which labels socket (SO) and shaft (S) cells, but does not co-localize with anti-Su(H), which labels the socket (F). (G) In ch organs, *atk* is expressed by the cap cell (CAP) based on *atk*-expressing cell position and co-localization with anti- α- Tub85E (arrowheads, G-G'). CA: cap attachment cell; N: neuron.

Similarly to ch organs, *atk* is not expressed by the neuron in es organs (Figure 1D-D''). Cell lineage studies suggest that the es organ equivalent of the cap cell of ch organs is the socket or

tormogen cell (Lai and Orgogozo 2004). This cell secretes the receptor lymph (Keil, 1997), with high potassium content and low calcium, and expresses the transcription factor *Suppressor of Hairless* (*Su*(*H*)) (Barolo *et al*. 2000). *atk* RNA, however, did not colocalize with anti-Su(H) (Figure 1F), suggesting that the *atk*-positive cell of es organs is not the socket cell. The other es accessory cells are the sheath or thecogen cell that encloses the cilium, and the trichogen or shaft cell which secretes the cuticular bristle shaft that is seated in the socket –in the es lineage, the cell corresponding to the ligament cell of ch organs enters apoptosis (Fichelson and Gho 2003)-. All cells of es organs express the transcription factor *cut,* yet after the in situ hybridization, anti-Cut antibody only labeled the shaft and socket cells. One of these cells expressed *atk* RNA (Figure 1E-E''), and since the socket cell was already ruled out as a candidate, we unambiguously identify the shaft cell as the *atk*-positive cell. Hence, *atk* is specifically expressed in certain accessory cells in type I sensory organs, i.e. the cap cells of ch organs and the shaft cells of es organs (Figure 1C).

Generation and expression pattern of *atk* mutant alleles: *atk* is located on the third chromosome at 77C3-C4 and is 10172 bp long. The gene *mag* is encoded in the opposite DNA strand within the first intron of *atk*. To generate mutations in the *atk* locus, we mobilized the P{EPgy2} element $Uhg8^{EY07139}$ that is inserted 170 bp downstream of the 3' end of *atk* (Figure 2A). Two hypomorphic alleles, atk^{8} and atk^{23}, were generated by imprecise excision of this P-element. Both alleles delete approximately half a kilobase from the 3' region of the gene; atk^{8} deletes 514 bp including 142 C-terminal amino acids and atk^{23} deletes 436 bp including 122 C-terminal amino acids (Figure 2A). Analysis of *atk* expression profile in atk^{8} and atk^{23} mutant embryos by in situ hybridization revealed that *atk* expression persists in the chemosensory organs of the embryonic head, but it is no longer detectable in the mechanosensory organs of the body segments (Figure 2C, data for atk^{8} not shown). Because

both mutations equivalently caused this partial loss of *atk* expression, we only used atk^{23} in subsequent experiments.

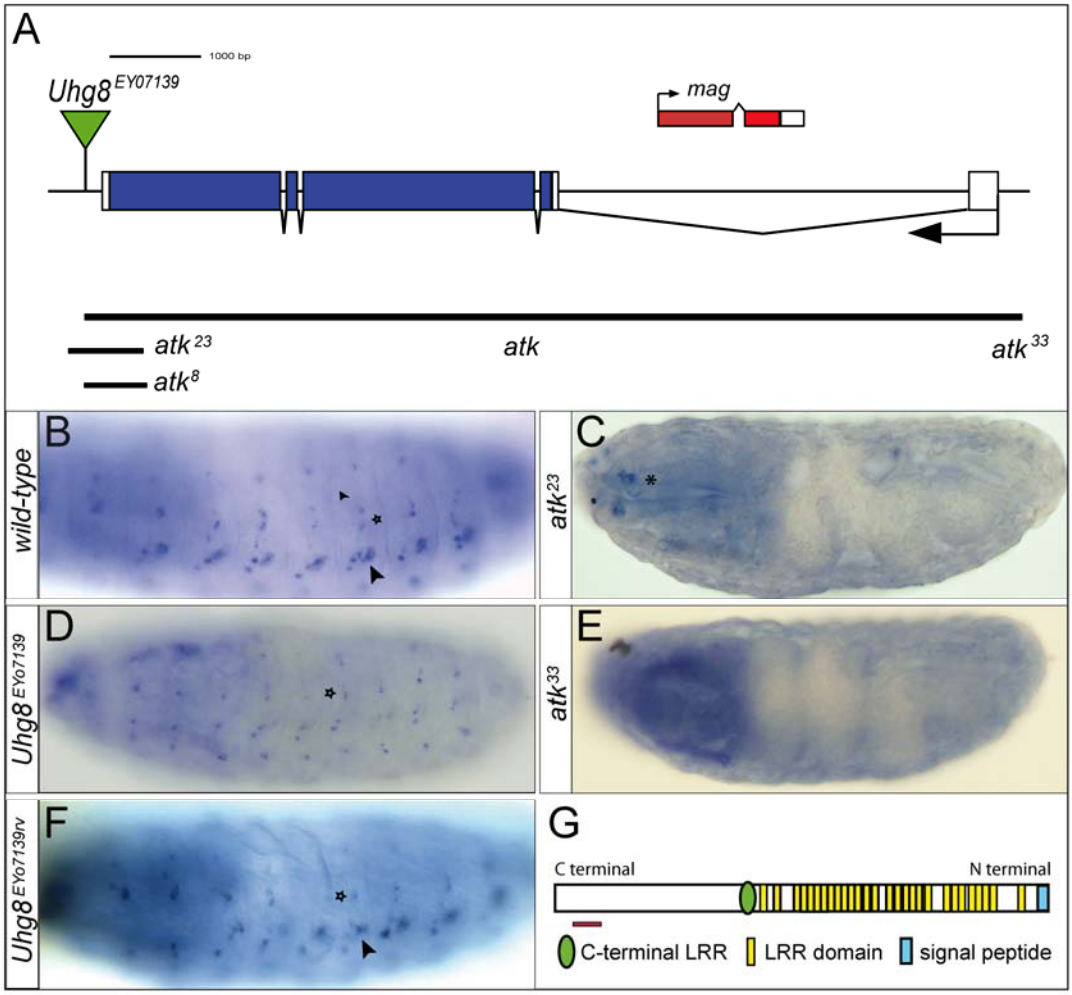

Figure 2. Generation of *atk* mutant alleles and anti-ATK antibody. (A) Schematic of *atk* locus. Shown are location of the P{EPgy2} element $Uhg8^{EY07139}$, extent of deletion in the alleles generated – hypomorphs atk^{8} and atk^{23}, and null allele atk^{33}–, and location of the gene *mag*. (B-F) Stage 16 embryos hybridized with an *atk* antisense RNA probe. (B) Wild type embryo showing hybridization in es organs (star) and ch organs (arrowhead). (C) atk^{23} mutant embryos lack *atk* expression in mechanosensory organs, but expression persists in chemosensory organs (asterisk). (D) Embryos homozygous for $Uhg8^{EY07139}$ lose *atk* expression in ch organs (arrowhead) but keep it in es organs (star). (E) atk^{33} homozygous embryos show no *atk* expression. (F) $Uhg8^{EY07139rv}$ show normal *atk* expression. (G) Predicted ATK protein with 29 LRRs. Red line indicates the region against which anti-ATK antibody was raised.

Even though a large number of P{EPgy2} element $Uhg8^{EY07139}$ excisions were analyzed, we did not obtain an *atk* null mutant. We therefore used P-element induced male recombination

(see Materials and Methods), which produced the null allele *atk*^{33} that contained a 18 kb deletion proximal to the P element insertion site, deleting also the genes *mag*, *CG42764* and *CG5955*. In situ hybridization of *atk*^{33} mutant embryos revealed no detectable expression of a*tk* (Figure 2E) indicating that *atk*^{33} is a bonafide null allele. All homozygous *atk* mutants are viable and fertile.

Partial loss of *atk* expression, as observed in hypomorphic *atk* mutants, also characterized the P{EPgy2} line *Uhg8*^{EY07139} that was used to generate the mutant *atk* alleles: judging from in situ hybridizations, *atk* expression persisted in head sensilla and es organs, but not in ch organs (Figure 2D). The P-element *Uhg8*^{EY07139} is not located within the *atk* coding region, pointing to an *atk* expression regulatory unit located at the *Uhg8*^{EY07139} insertion site. Removing the P-element *Uhg8*^{EY07139} in the line *Uhg8*^{EY07139rv} restored the complete pattern of *atk* expression observed in wild type embryos (Figure 2F).

ATK localizes to the supporting ECM of type I sensory organs: The predicted ATK protein contains 1535 amino acids (Figure 2G), including 29 predicted LRRs. Sequence analysis identifies a signal peptide in amino acids 1-19 (Signal 4.0) and a highly hydrophobic short C-terminal sequence (ProtScale, Swiss Institute of Bioinformatics, Fribourg, Switzerland). Thus, ATK could either be secreted, or attached to the external membrane surface of the *atk*-expressing cell through its carboxy tail. We generated an antibody against the C-terminal region of ATK (amino acids 1403-1523), which is free of LRR domains, to avoid cross-reaction of antibody recognition sites with other LRR-containing proteins. Antibody specificity was confirmed using Western Blot (Figure 3D). The antiserum labeled all mechanosensory ch and es organs in immunostained embryos, producing a punctate staining adjacent to -and distally from- the tip of the sensory cilium (Figure 3) This staining persists in late stage 17 embryos, when *atk* is no longer being transcribed, but disappears

during larval stages (Figure 3E,F). Therefore, ATK protein is only present in a narrow time window, which coincides with the late development of the larval sensilla.

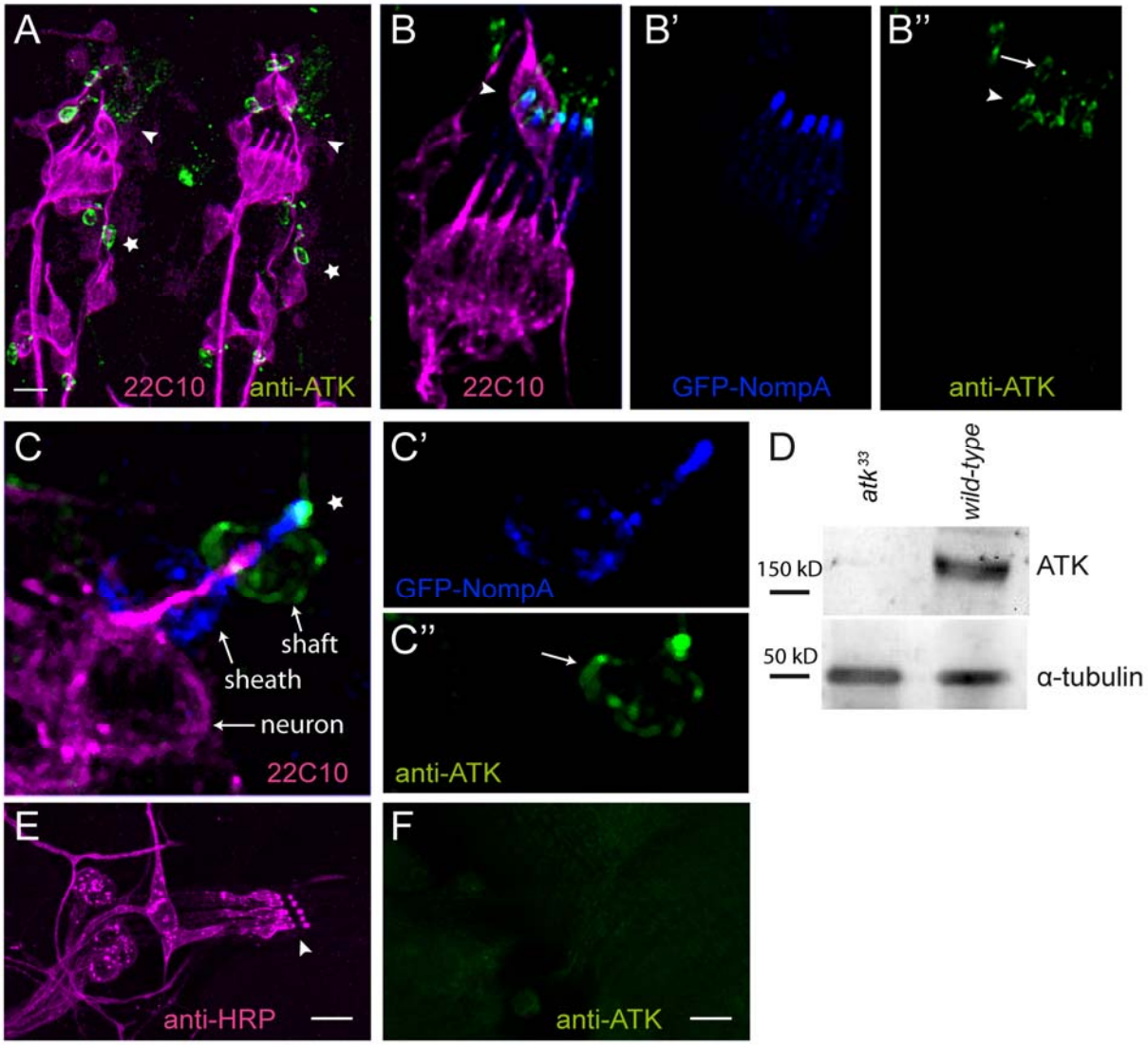

Figure 3. ATK localizes to the distal region of the dendritic cap in late embryonic mechanosensory organs. (A) Stage 16 embryonic hemisegments immunostained with the neuronal marker mAb22C10 (magenta) and with anti-ATK (green). ATK localizes apically to the sensory cilia in ch (arrowhead) and es (star) organs. (B-C) Detailed view of embryonic mechanosensory organs additionally showing GFP-NOMPA (blue) in the dendritic cap. In lch5 (B) and es (C) organs, ATK localizes to the distal region of the dendritic cap (arrowhead in B, star in C), where it partially overlaps with GFP-NOMPA. The cytoplasm of cap and shaft cells are also stained (arrows). (D) Western blot with anti- ATK antibody. (E-F) ATK protein is not present in larval mechanosensory organs. Second instar larval lch5 organ stained with anti-HRP (E) shows no detectable anti-ATK staining (F). Scale bars indicate 10 µm.

We double immunostained embryos with GFP-NOMPA, a functional fusion protein reporting the dendritic cap localization of NOMPA, which is produced by the scolopale cell of ch organs and the thecogen (sheath) cell of es organs (Chung *et al*. 2001). Co-localization of ATK and GFP-NOMPA in both ch (Figure 3B) and es (Figure 3C) organs placed ATK to the dendritic cap. ATK antibody also stained the cytoplasm of those accessory cells (cap or shaft) that express *atk*. Consistent with the hybridization signals, no antibody staining was observed in multidendritic type II sensory organs or outside the PNS (not shown).

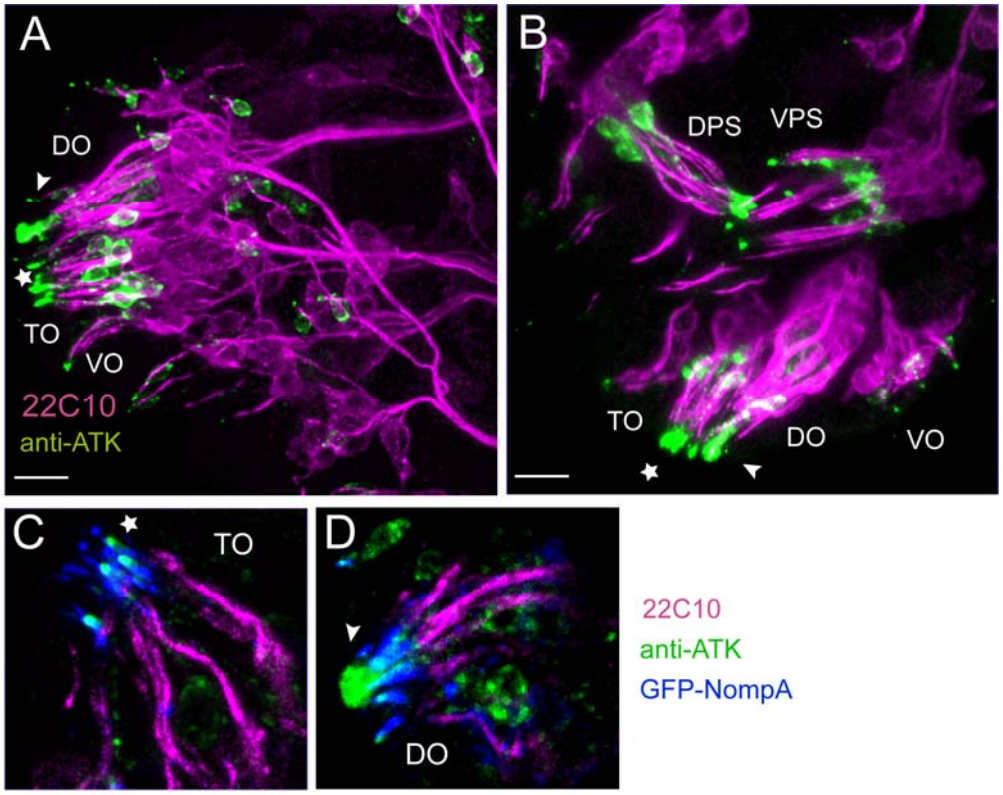

Figure 4. ATK localizes to a supporting ECM in late embryonic chemosensory organs. (A-B) Stage 16 embryonic chemosensory organs immunostained with the neuronal marker mAb22C10 (magenta) and anti- ATK (green). DO: dorsal organ, TO: terminal organ, VO: ventral organ; DPS: dorsal pharyngeal organ; VPS: ventral pharyngeal organ. In DO (arrowhead) and TO (star), ATK localizes to the tip of the chemosensory cilia. Cell bodies of *atk*-expressing cells are also stained. Scale bars indicate 10 µm. (C-D) Detailed view of TO (C) and DO (D) additionally showing GFP-NOMPA (blue). (C) In TO, ATK localizes at the tip of the GFP-NOMPA region (star), where gustatory neurons are exposed to the environment. (D) In DO, ATK forms a vacuole-like structure in the center of the cilia cluster (arrowhead).

Strong anti-ATK staining was seen in embryonic chemosensory organs (Figure 4). The larval head chemosensory system includes three pharyngeal organs (DPS, VPS, PPS) that may serve gustation and mechanosensation (Singh 1997), as well as three external sensory organs, the dorsal (DO), the terminal (TO) and the ventral (VO) organs. Each of these organs consists of several sensilla, with each sensillum comprising one to nine sensory neurons and three accessory cells. The DO ends in a multiporous "dome" suggesting an olfactory function, and is surrounded by six peripheral sensilla with gustatory function. The TO and VO respond to tastants only and are characterized by a terminal pore (Stocker 2008). We found that ATK is present in all these organ types in stage 16 embryos.

Although the neuronal composition of the larval chemosensory system is well established in *Drosophila* (Grillenzoni *et al*. 2007; Stocker 2008), much less is known about the accessory structures. Studies in *Musca domestica* show that the DO is filled by a large-fluid containing vacuole, from the proximal region where the cellular bodies lie, to the distal lumen of the dome. Microvilli from the trichogen and tormogen cells protrude into the vacuole and are thought to secret compounds into it. The sensory cilia surround the vacuole and branch after reaching the dome, but are not exposed to the exterior, so that to reach their olfactory receptors, the chemicals should cross pores that go through the dome (Chu-Wang and Axtell 1971). Our characterization of ATK localization in the *Drosophila* chemosensory sensilla extends previous descriptions and shows that their structure is remarkably similar to that reported in *Musca domestica* (Chu-Wang and Axtell 1971; Chu-Wang and Axtell 1972a; Chu-Wang and Axtell 1972b). The distribution of the anti-ATK antibody in the late embryonic DO, where it labels a vacuolar-like elongated structure in the middle of the cluster of sensory cilia (Figure 4D), suggests that ATK is probably secreted by the trichogen cell into the central vacuole during the late stages of cilium growth. The protruding appearance of ATK-localizing region suggests that it might fill the dome cavity. On the other hand, in the

late embryonic TO and VO, ATK localizes to the supporting ECM that enwraps each cilium up to the point where the cilium enters the cuticular pore and exposes itself to the environment (Figure 4C) (Chu-Wang and Axtell 1972a; Chu-Wang and Axtell 1972b). This is also the case in the gustatory organs from the pharyngeal region. Strong expression was also seen in the anal sensory cones in stage 16 embryos (not shown).

Lack of *atk* causes defects in the stretching of the chordotonal organs: Since ATK transiently localizes to the supporting ECM of ciliated mechanosensory organs, we analyzed *atk* mutant embryos for morphological defects of the lch5 organ, the most prominent embryonic ch organ.

To visualize the sensory neurons and cap cells, we double stained late stage embryos with either neuronal marker mAb22C10 or anti-HRP, and with anti-α-Tub85E (Matthews *et al*. 1990). The lch5 organ comprises 5 scolopidia that are arranged in a row with their cilia pointing to the dorsal posterior region of the embryo (Figure 5A,C). Their tips penetrate the cap cells and contact them through the dendritic cap (Figure 5E). Cilia are parallel and equidistant to each other. All *atk* mutant alleles showed defects in the morphology of embryonic lch5 organs (Figure 5B,D). Both cilia and neuronal cell bodies were affected. Cilia were not parallel to each other, but pointed out in different directions (Figure 5B,D). However, staining mutant embryos with anti-HRP, a neuronal marker that stains the cilia up to their tips (mAb22C10 stains only the inner dendritic segment that ends at the basal body, compare Figure 5C and 5E), revealed that *atk* mutant cilia still contact the cap cell (Figure 5F). Neuronal cell bodies also appeared frequently disorganized in *atk* mutant lh5 organs (Figure 5B,D). We considered a lch5 organ to be 'artichoke-like' when both the orientation of its cilia and the arrangement of its cell bodies were defective. A morphological blind scoring showed that defects were equally penetrant in hypomorphic atk^{23} and null atk^{33} mutants, as

well as in homozygous *Uhg8*^{EY07139} mutants (Figure 5G), consistent with the complete loss of *atk* expression in ch organs observed in these strains (Figure 2D). Removing the P-element in *Uhg8*^{EY07139rv} flies fully rescued the morphological defects (Figure 5G).

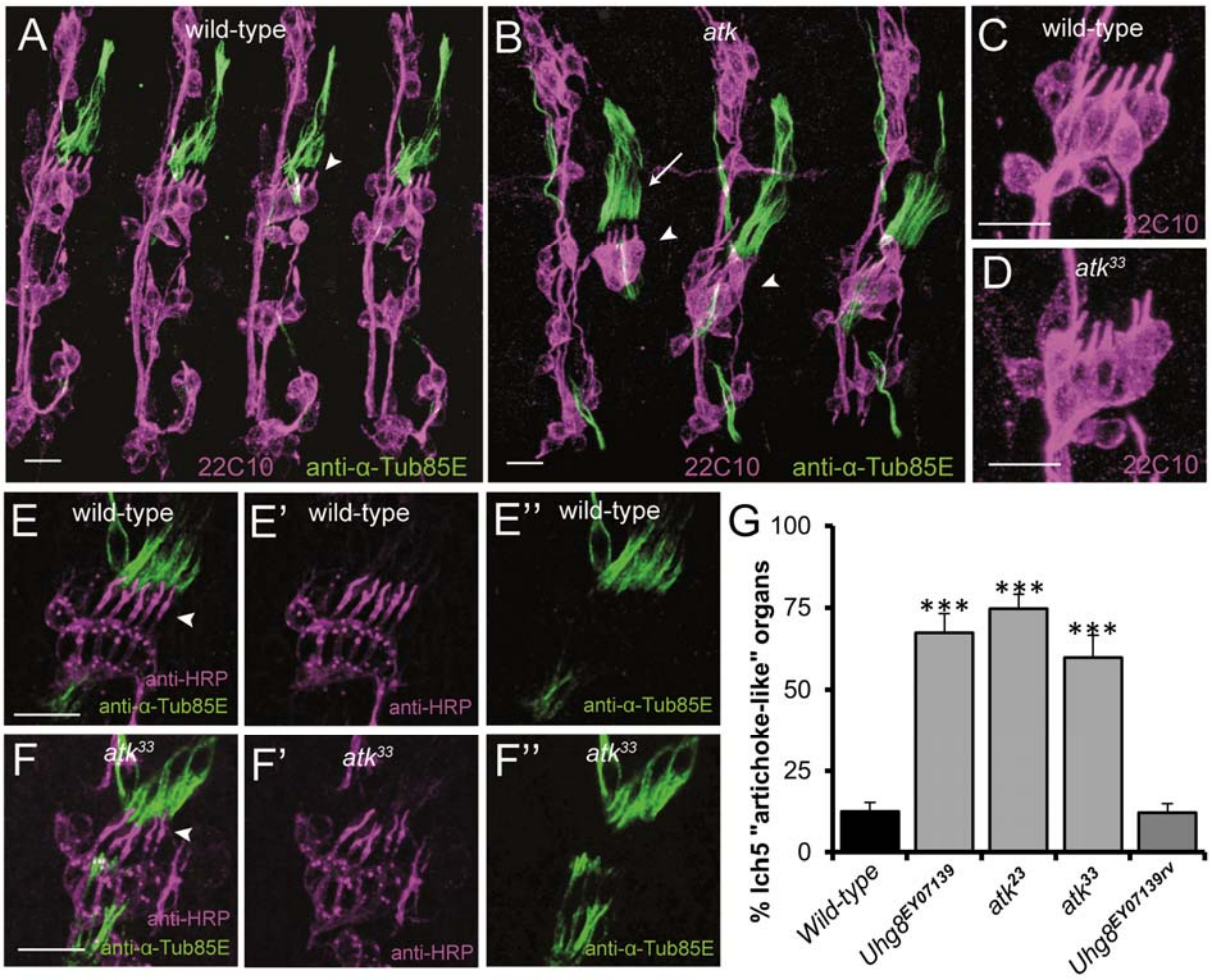

Figure 5. *atk* mutants show abnormal embryonic lch5 organ morphology. (A-D) Stage 16 embryonic hemisegments stained with neuronal marker mAb22C10 (magenta, labeling cell body and inner dendritic segment) and anti-α-Tub85E (green), which labels all ch organ accessory cells except the scolopale cell. (A,C) In wild type lch5 organs, sensory cilia are parallel and point to the same direction (arrowhead). Neuronal cell bodies are aligned in a row. (B,D) In *atk*^{33} embryos, many lch5 organs show an "artichoke-like" phenotype with non-parallel dendrites and misaligned cell-bodies (arrowheads in B). Cap cells also show defects (arrow in B). (E,F) Lch5 organs of stage 16 embryos, stained with anti-HRP (magenta) that labels the entire cilium, and anti-α-Tub85E (green). The cilium tip contacts the dendritic cap in both wild type (E, arrowhead) and *atk*^{33} mutants (F, arrowhead). Scale bars indicate 10 μm. (G) Percentage of "artichoke-like" lch5 organs per hemisegment and genotype. Embryos were stained with mAb22C10 to label the sensory neurons and scored blind to genotype. Significant difference was established by Kruskal-Wallis Test followed by a non-parametric post-hoc test in order to compare each genotype with wild type. ***, $P < 0.001$.

Despite its transient expression, ATK is required for the correct embryonic development of chordotonal organs which, although grossly normal, often show misplacement of cilia and/or neuronal soma. We analyzed the consequences of these defects at later stages, during the second and third larval instars (Figure 6). Cap and ligament cells of larval lch5 were normally stretched in *atk* mutant larvae (not shown), but, as in embryos, neuronal cilia were frequently disorganized (compare Figures 6B and 6D). Additionally, anti-HRP signal was reduced in all lch5 mutant cilia and severely mislocalized, even though neuronal soma had similar levels of immunoreactivity (Figure 6). These alterations suggest functional defects in mutant larval mechanosensory receptors.

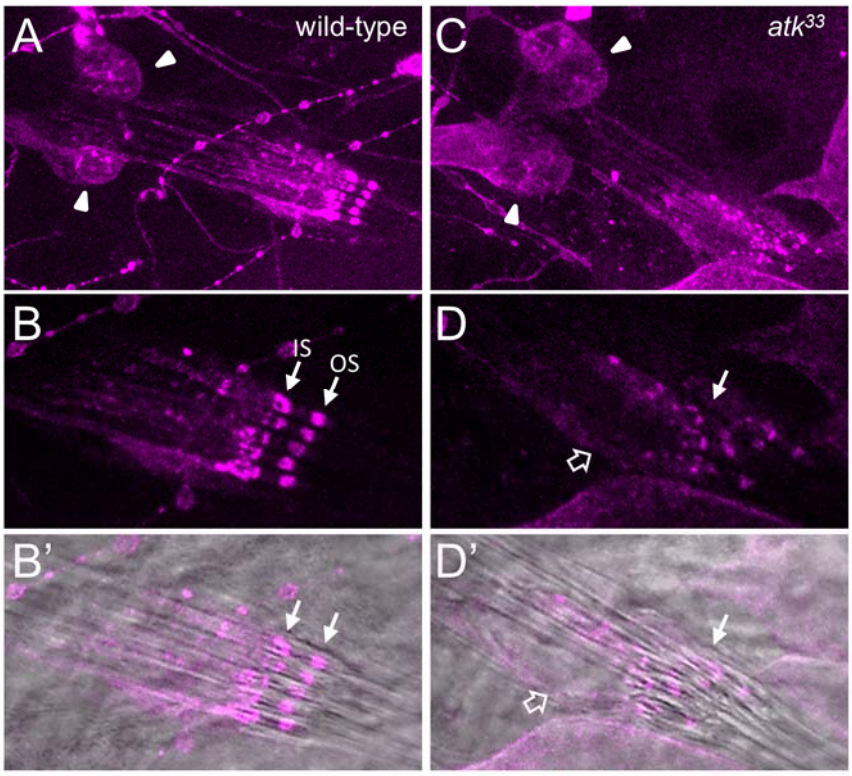

Figure 6: *atk* mutant larvae show abnormal lch5 organ morphology and altered anti-HRP signal in cilia. Third instar larval lch5 organs stained with anti-HRP (magenta) in control (A,B) and atk^{33} mutants (C,D). (A,C) General view of neuronal cell bodies (arrowheads) and cilia. (B,D) High magnification of cilia showing anti-HRP signal, alone (B,D) or merged with a bright field image (B',D'). In control lh5 organs (B) cilia are parallel and aligned, with anti-HRP signal concentrated in two bands corresponding to inner (IS) and outer (OS) segments (Ma and Jarman 2011). In *atk* mutants (D), alignment of cilia is lost (open arrow points to a cilium that is bent and separated from the others), and distribution of anti-HRP immunoreactivity severely disrupted.

***atk* mutant larvae exhibit locomotion defects:** Many mutations affecting larval ch organs cause locomotion defects. Although muscle contractions that direct larval locomotion are controlled by the central nervous system, feedback from ch organs is required to coordinate the larval movement (Caldwell *et al*. 2003; Cheng *et al*. 2010). To test whether *atk* mutants display impaired locomotion, we assayed the crawling behavior of third instar larvae. Consistent with the anatomical defects in lch5 organs, all *atk* mutants exhibited severe defects in larval locomotion: the number of direction changes was significantly increased (Figure 7A) and the total path length was significantly reduced (Figure 7B). In the mutants, phases of translational movement were scarce and short, and larvae often stalled at the same point turning their bodies several times in different directions. The severity of the defect was similar in all *atk* alleles, including $Uhg8^{EY07139}$. The fact that $Uhg8^{EY07139}$ homozygous larvae, which do no longer show detectable *atk* expression in ch organs but keep a detectable expression in es organs, do also show locomotive defects, confirms previous works that propose ch organs –by contrast to es organs– as major contributors to the locomotive sensory feedback (Caldwell *et al*. 2003; Cheng *et al*. 2010).

Locomotion defects were fully rescued in $Uhg8^{EY07139rv}$ revertants, highlighting the importance of ATK and ch organs for coordinated larval movements.

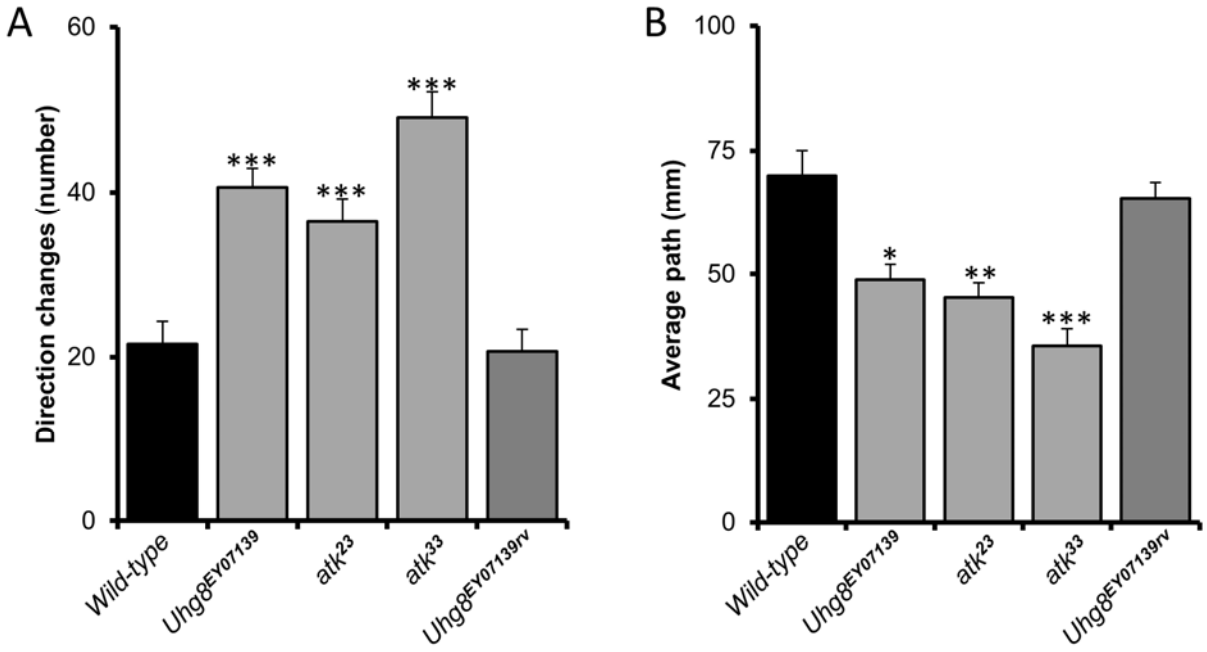

Figure 7. *atk* mutants show larval locomotion defects. Locomotion was scored for 100 seconds by measuring the total number of direction changes (A) and the average path length (mm) (B). Means are represented. Error bars indicate s.e.m. Significant difference with respect to wild type was established by Bonferroni corrected t test. *, $P < 0.05$; ***, $P < 0.005$.

ATK is required for an adequate larval response to sucrose: ATK is transitorily present in all gustatory organs of *Drosophila* embryos. In the main embryonic gustatory organs, VO and TO, ATK localizes just underneath the base of the pore through which each gustatory cilium projects (Figure 4C). Our attempt to detect anatomical defects in *atk* mutants was unsuccessful because of the lack of a simple stereotyped cilium pattern in chemosensory organs that renders the identification of anatomical defects a difficult task.

Therefore, to assess the requirement of ATK in chemosensation, we tested the response of larvae to sucrose, which induces positive chemotaxis (Miyakawa 1982). A response index (RI) was calculated after letting larvae choose for 15 minutes between 1% agarose, and 1% agarose supplemented 0.5 M sucrose. Among the *atk* mutant alleles, only atk^{33} null mutants

showed a reduced response to sucrose; neither the hypomorphic *atk*^{23} and *Uhg8*^{EY07139} larvae nor the *Uhg8*^{EY07139rv} revertants displayed chemosensory defects (Figure 8). These results seem consistent with the finding that *atk* expression in gustatory organs is abolished in the null mutants but persists in hypomorphic animals. However, we should be cautious interpreting these results, because we cannot exclude the effect of additional genes removed by the *atk*^{33} deletion. Our attempts to rescue the anatomical and behavioral phenotypes with a Gal4-driven *UAS-atk* construct failed (not shown), probably as a consequence of the inability to reproduce the transient expression of the gene. Still, the gustatory impairment is consistent with ATK expression in gustatory sensilla and suggests that the ECM also plays essential roles in this type of ciliated sensory organs.

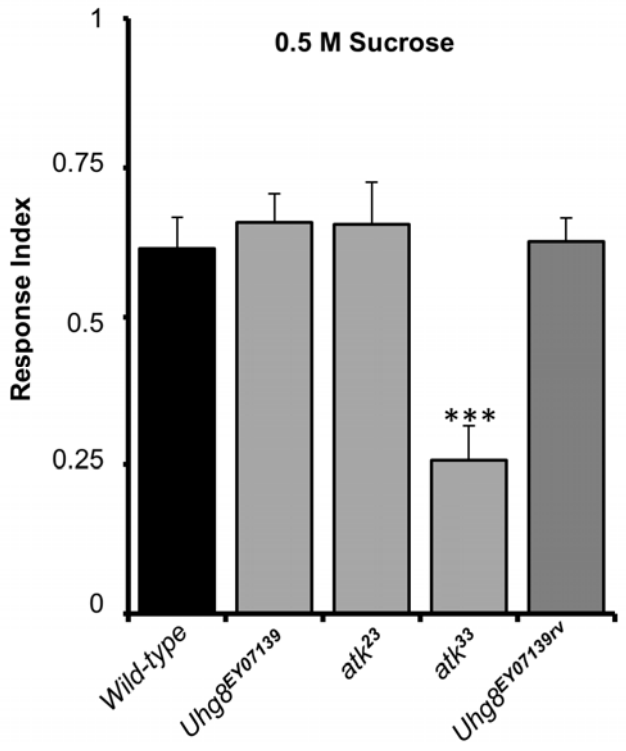

Figure 8. *atk* mutant larvae show a significantly reduced response to sucrose. Response index for election between 1% agarose and 1% agarose + 0.5 M sucrose. *atk*^{33} null mutants show defects in chemosensation. *atk*^{23} hypomorphs and *Uhg8*^{EY07139} homozygous, which keep *atk* expression in chemosensory organs, do not show defects. Therefore ATK seems to be required for chemosensation, although an effect of additional genes deleted by the *atk*^{33} deficiency cannot be ruled out. Error bars indicate s.e.m. Significant difference with respect to wild type was established by Bonferroni corrected t test. ***, $P < 0.001$

DISCUSSION

In this study we have tried to shed some light on the function of the supporting ECM in sensory organs by characterizing the novel secreted LRR-protein Artichoke in *Drosophila melanogaster*. ATK transiently localizes to the most distal region of the ECM in ciliated sensory organs undergoing their late embryonic developmental steps. The presence of ATK in all ciliated sensory organs, its conservation among organisms with primary cilia (Avidor-Reiss *et al.* 2004) and the behavioral defects shown by *atk* mutants suggest a role for ATK in cilia assembly and function. Our work additionally suggests that a proper coupling between the sensory cilium and the supporting ECM is also essential for the correct assembly of chemosensory organs. To our knowledge, these results provide the first experimental evidence for an ECM role in *Drosophila* chemosensation.

ATK is a component of the sensilla ECM secreted by the Cap and Shaft cells: During the late stages of sensillum morphogenesis, *atk* is expressed in all embryonic ciliated sensory organs, including both chemo- and mechanoreceptors. ATK is the first molecule to be described that is secreted into the ECM by the shaft cell in es and chemosensory organs and by the cap cell in ch organs. In *Musca*, the matrix of the chemoreceptors is thought to be secreted by the trichogen and tormogen cells (Chu-Wang and Axtell 1971), as is the case for ATK. In *Drosophila*, this matrix was previously believed to be secreted only by the thecogen or scolopale cell (Hartenstein 1988; Carlson *et al*. 1997b). Our data provide the first direct evidence for assigning to cap and shaft cells a role in the secretion of essential components of the extracellular matrix, reinforcing the importance of these accessory cells for the development of the ciliary sensilla (Mrkusich *et al*. 2010).

The observation that the mutant alleles differentially affect *atk* expression in ch, es, and chemosensory sensilla suggests the presence of organ-specific regulatory units that control *atk*

transcription in different types of sensory organs. A similar phenomenon has been described for the ciliogenesis regulator Regulatory Factor X (RFX): although RFX is expressed in both ch and es organs, it is activated by different enhancers in both organ types, and seems to be a direct target of the proneural gene *atonal* in ch organs but an indirect target of the *scute* proneural gene in es organs (Cachero *et al*. 2011). The existence of different transcriptional regulatory elements could explain the temporal and quantitative variations in *atk* expression observed in mechanosensory ch, es and chemosensory sensilla.

ATK is an ECM protein implicated in the late morphogenesis of ciliated sensory organs: Since ATK is present in all embryonic ciliated sensory organs in *Drosophila*, it seems likely that it was already present in the ancestral ciliated sensory organ and has been preserved in the diversified sensory organs to play a similar, essential function (Lai and Orgogozo 2004). Previous support for an evolutionary conserved association between ATK and cilia came from the study by Avidor-Reiss *et al*. 2004, in which several ciliogenesis genes were identified. Through comparative genomic analysis of ciliated and non-ciliated organisms, *atk* was shown to be specifically present in ciliated organisms, including *Trypanosoma brucei, Drosophila* and humans. Because all the *atk*-containing organisms assemble at least some of their cilia through compartmentalized ciliogenesis –the process in which the cilium protrudes gradually from the basal bodies located under the plasma membrane–, ATK was classified as a compartment gene (Avidor-Reiss *et al*. 2004). However, our results show that ATK is not strictly required for cilium growth, because *atk* mutants apparently form cilia of normal length. Instead, we propose that ATK-like proteins are extracellular proteins involved in connecting cilia to accessory structures during late organ refinement.

Our data showing partial extracellular colocalization of ATK and NOMPA indicate that ATK transiently localizes to the site of contact between the cilium and distal accessory cells in

embryonic mechanosensory sensilla, and are consistent with a similar distribution in chemoreceptors (Hartenstein 1988; Hartenstein 2005; Keil 2012; Yack 2004). Although ch organs reach their basic final shape and position at stage 15, further refinement occurs during later stages, at the time when ATK expression peaks (Carlson *et al*. 1997b; Inbal *et al*. 2003; Kraut and Zinn 2004; Hartenstein 2005). Several studies suggest the importance of the accessory cells in this final process of organ refinement: in the v'ch1 chordotonal organ (Mrkusich *et al*. 2010), the positioning of the growing ciliary dendrite has shown to result from pulling forces that the dorsally extending cap cell exerts on the dendrite tip; and in the lch5 organ, the interaction between ligament and ligament attachment cells ventrally straightens the whole organ (Inbal *et al*. 2004; Klein *et al*. 2010). Thus, to ensure proper spatial and temporal transmission of the morphogenetic forces, the different components of the sensillum must interact adequately. The finding that lack of ATK during just these late stages has dramatic consequences on the overall morphology and functioning of the organ may reflect the requirement of a specialized ECM that maintains the apical components intimately communicated by conferring particular adhesive and signaling properties. Our results further suggest that the ECM in chemoreceptors not only plays a physiological function (Ebbs and Amrein 2007), but it is required for the proper maturation also of these organs.

The temporal and spatial localization of ATK suggests a developmental role for this protein in the very late morphogenetic events leading to the assembly of ciliated sensory sensilla. The role that ATK is playing in other ciliated organisms remains an interesting question. The fact that an extracellular molecule expressed in *Drosophila* by and accessory cell rather than by the ciliated cell is conserved among ciliated organisms is intriguing, especially for unicellular organisms like *Trypanosoma brucei* (Avidor-Reiss *et al*. 2004). However, this organism uses its flagellum to attach to the gut of the tsetse fly prior to infection (Dyer *et al*. 2013), and an

adhesion molecular machinery is required for this process. A speculative, attractive hypothesis is that adhesion mechanisms that facilitate the connection of cilia are conserved along evolution independently of whether their purpose is infection or properly connecting sensory cilia to the stimulus detecting apparatus.

In summary, our work shows that terminal differentiation of ciliated sensory organs in *Drosophila* is finely tuned by proteins like ATK that, although transiently expressed, have long-lasting functional effects. As far as we know, there is only one other protein that has a comparable influence: the β3-tubulin, which is expressed transitorily by the cap cell in ch organs at the same stages as *atk*, seems to confer permanent features of microtubule organization that persist in the fully differentiated cell, even after β3 is no longer present (Dettman *et al*. 2001). This highlights the importance of accessory cellular and extracellular structures not only for the process of sensory transduction, but also for the correct organ maturation.

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