

# **Obstructor-A organizes matrix assembly at the apical cell surface to promote enzymatic cuticle maturation in *Drosophila***

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**Running title:** aECM assembly at barrier tissues

## **Key words**

Membrane; extracellular matrix; molecular genetics; chitin; functional genomics; *Drosophila*

## Capsule

**Background:** The apical extracellular matrix (aECM) protects against environmental stresses that attack organisms throughout lifetime.

**Results:** Epidermal cells secrete Obstructor-A into a core-organizer region for controlling aECM assembly at the apical cell surface.

**Conclusion:** Normal aECM assembly is mediated by Obstructor-A and regulates cuticle stability at the epidermis.

**Significance:** Cuticle formation of the insect epidermis is genetically conserved.

## ABSTRACT

Assembly and maturation of the apical extracellular matrix (aECM) is crucial for protecting organisms, but underlying molecular mechanisms remain poorly understood. Epidermal cells secrete proteins and enzymes that assemble at the apical cell surface to provide epithelial integrity and stability during developmental growth and upon tissue damage. We analyzed molecular mechanisms of aECM assembly and identified the conserved chitin-binding protein Obstructor (Obst)-A as an essential regulator. We show in *Drosophila* that Obst-A is required to coordinate protein- and chitin-matrix packaging at the apical cell surface during development. Secreted by epidermal cells, the Obst-A protein is specifically enriched in the apical assembly zone where matrix components are packaged into their highly ordered architecture. In *obst-A* null mutant larvae, the assembly zone is strongly diminished resulting in severe disturbance of matrix-scaffold organization and impaired aECM integrity. Furthermore, enzymes that support aECM stability are mislocalized. As a biological consequence, cuticle architecture, integrity and function are disturbed in *obst-A* mutants finally resulting in immediate lethality upon wounding. Our studies identify a new core-organizing center, the assembly zone that controls aECM assembly at the apical cell surface. We propose a genetically conserved molecular mechanism by which

**Obst-A forms a matrix-scaffold to coordinate trafficking and localization of proteins and enzymes in the newly deposited aECM. This mechanism is essential for maturation and stabilization of the aECM in a growing and remodeling epithelial tissue as an outermost barrier.**

A critical feature for organisms is the formation and integrity of the outermost barrier to protect their body against dehydration, invading pathogens and toxic environments. Epidermal, gut and tracheal cells deposit aECM components at the apical surface where they need to assemble. The insect aECM provides an exoskeletal cuticle that is in contact with the surrounding environment for protection against lethal infections and other environmental stresses. The developmental progress of larvae requires repetitive molting (ecdysis), the replacement of old by new cuticles, to accommodate increasing body size. Therefore mechanisms that coordinate assembly and maturation of all new cuticles are critical for barrier integrity. Even though production and degradation are well described in insects (1-6), the regulatory processes of forming compact aECM barriers remain poorly understood.

Chitin synthases are active at the apical cell membrane where they produce nascent chitin polymers (1-3). Deposited at the apical cell surface chitin polymers spontaneously form microfibrils of varying length and diameter (3). A systematic assembly of the chitin based aECM generates a prominent and stable part of the growing exoskeletal cuticle (7). Therefore, during late larval stages existing compact cuticles need to stretch when new matrix is deposited at the apical cell surface for increasing overall cuticle thickness and stability. Additional enzymatic modifications, such as deacetylation, promote essential cuticle maturation of the aECM (8-11). Chitin deacetylation fundamentally improves chemical and physical matrix properties of organs, tissues and even engineered materials (12,13). However, mechanisms that control the aECM formation during larval molting, and at larval intermolt, when the cuticles receive their compact organization but need to be flexibly stretched (14), are yet unknown.

Obst-A is a member of the *obstructor* multigene family, which is highly conserved among arthropods and expressed in chitin producing epithelia (15). Homologous *obstructor* genes were recently also identified in beetles (Cuticular Proteins Analogous to Peritrophins 3 (CPAP3)), mosquitos and other insects (16-18). In *Drosophila* the Obst-A chitin-binding protein interacts with the deacetylation domain protein Serpentine (Serp) and the chitin protector Knickkopf (Knk) to prevent premature degradation of the tracheal matrix in late embryos (19). Here we define a new Obst-A dependent chitin-matrix organizing center in the epidermis, the yet uncharacterized assembly zone, which links the epidermal epithelium with the formation of a highly organized cuticle-exoskeleton. The role of the assembly zone is to modulate aECM assembly at the cell surface for protecting animals during molting and upon wounding.

## EXPERIMENTAL PROCEDURES

### Fly stocks and genetics

Flies were derived as described below: white<sup>1118</sup> (w-; here referred to as wild type), *btl*GAL4, 69BGal4 driver lines and *knk*<sup>10192</sup> (Bloomington stock center), *mega*<sup>VE</sup> (20), *obst-A*<sup>d03</sup> (19), *serp-verm* double mutant (10), UAS-RNAi-*serp*, UAS-RNAi-*verm* and UAS-RNAi-*knk* fly lines were obtained from the Vienna stock center. To distinguish mutants from others, balancer chromosomes were used: FM7i,P{ActinGFP} or TM3,P{GAL4*twi*.G} 2,3P {UAS-2xEGFP} or TM3*serP*{w[+m]*hsp70*:GAL4}P{w[+m]UAS:GFP}. The absence of GFP expression was used as a marker to identify larvae carrying hemizygous and homozygous mutant alleles. Transheterozygous mutants were generated by mating of *obst-A*/FM7i,P {ActinGFP} heterozygous females with either *serp,verm*/TM3,P {GAL4*twi*.G} 2,3P {UAS-2xEGFP} or *knk*/TM3*serP*{w[+m]*hsp70*:GAL4}P{w[+m]UAS:GFP} heterozygous males. The non-GFP offspring of first instar larvae, collected and tested for cuticle integrity, include 50 % of *obst-A* hemizygous and 50 % of transheterozygous mutants. Due to early larval lethality of *obst-A* hemizygous null mutants, analyzed non-GFP offspring collection for immunofluorescent

stainings at second and third instar larvae only contained transheterozygous mutants. Crosses of 69BGal4 driver flies and UAS-RNAi-*knk*, -*serp*, and -*verm* flies, respectively, result in RNAi-mediated knockdown in the offspring epidermis.

### Antibodies and microscopy

Larvae were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in JB-4 Plus (Polysciences, Warrington, USA). Polymerized blocks were cut in 7 µm sections (Ultracut E, Reichert-Jung, Solms, Germany). Sections were rehydrated and subjected to an antigen-retrieval protocol in 10 mM sodium citrate, pH 6.0, at 65 °C depending on the primary antibody for 15 min (α-Obst-A, α-Knk) or 1 h (α-Serp, α-Verm) and incubated with 0.001 % trypsin in 0.05 M Tris-HCl, pH 8.0 at 37 °C for 1 h. Sections were blocked in PBS +10 % Donkey Serum for 30 min and stained overnight at 4 °C with Alexa-488 conjugated chitin-binding probe (Cbp; 1:100; New England Biolabs, Ipswich, USA), which selectively binds chitin. The Alexa-633 conjugated Wheat Germ Agglutinin (WGA; 1:250; Molecular Probes, Carlsbad, USA) is a lectin, which is able to react with internal sugar residues of glycoproteins and selectively recognizes N-acetylneuraminic acid and N-acetylglucosamine. WGA has a very strong affinity to chitin and was used to label the apical cell surfaces in *Drosophila* embryos and larvae (19,21-23). Embryo fixation and antibody stainings were performed as described previously (19,21,22).

Used antibodies: α-Spectrin (1:10, mouse, DSHB), Knk (1:333; rabbit) (24), Obst-A (1:300; rabbit) (19), Serp (1:175; rabbit) and Verm (1:175; rabbit) (10). Primary antibodies were detected by secondary antibodies linked with fluorescent dyes (Dianova, Hamburg, Germany and JacksonImmuno, Baltimore, USA) and mounted in Vectashield (Vector Laboratories, Burlingame, USA). For Z-stack analysis sequential scans were taken with Zeiss LSM710/LSM780 microscopes (Carl Zeiss, Göttingen, Germany) and a 63x LCI Plan Neofluar objective. The pinhole was adjusted to "airy unit 1" and standard settings were used. Images were cropped in ImageJ and Adobe Photoshop CS6 and figures designed with Adobe Illustrator CS6.

### Ultrastructure analysis

Larvae were placed on a 150  $\mu\text{m}$  flat embedding specimen holder (Engineering Office Wohlwend, Sennwald, Switzerland) and frozen in a Leica HBM 100 high pressure freezer (Leica Microsystems, Wetzlar, Germany). Automatic Freeze Substitution Unit (AFS) (Leica) was used for embedding of the vitrified samples. Substitution was performed at  $-90^\circ\text{C}$  in a solution containing anhydrous acetone, 0.1 % tannic acid and 0.5 % glutaraldehyde for 72 h and in anhydrous acetone, 2 %  $\text{OsO}_4$ , 0.5 % glutaraldehyde for additional 8 h. After 18 h of incubation at  $-20^\circ\text{C}$ , warmed ( $4^\circ\text{C}$ ) samples were washed with anhydrous acetone and embedded in Agar 100 (Epon 812 equivalent). Images were taken with a Philips CM120 electron microscope (Philips Inc; TemCam 224A slow scan CCD camera; TVIPS, Gauting, Germany). Indirect detection of chitin was carried out on Epon embedded samples as previously described by Moussian et al. (25) using immunogold-labeling of WGA recognizing chitin. WGA labels colloidal gold, an electron dense marker used to study chitin localization in ultrastructure analysis (26). WGA (5  $\mu\text{g}/\text{ml}$ , Vector Laboratories, Burlingame, USA) was detected by a rabbit-anti-biotin antibody (3.9  $\mu\text{g}/\text{ml}$ , Rockland, Gibbertsville, USA) and Protein A gold (10 nm, 1:100, G. Posthuma, Utrecht, Netherlands). As negative control immunogold-labeling without WGA was performed, as well as without primary antibody. These negative controls showed no significant unspecific labeling (data not shown).

We performed *Obst-A* immunogold-labeling on ultrathin sections of HM20 embedded samples as follows. The high pressure frozen larvae were substituted using the Leica AFS for 57 h at  $-90^\circ\text{C}$  in 1% uranyl acetate in acetone. After further incubations at  $-60^\circ\text{C}$  and  $-25^\circ\text{C}$  the samples were infiltrated with HM20 resin (Polysciences, Warrington, USA) and polymerized using UV light at  $-45^\circ\text{C}$  for 48 h. Immunogold-labeling of *Obst-A* was performed using 1:100 diluted *Obst-A* antibody and Protein A gold (10 nm, 1:50). For better visibility of the gold-labeling pictures were taken of not counterstained sections using a Philips CM120 electron microscope (Philips Inc; TemCam F416 CMOS camera; TVIPS, Gauting, Germany).

### Cuticle integrity test, survival assay, body size

Cuticle integrity was tested with a thin glass needle. Mid first instar larvae were laterally pricked at the posterior part and hemolymph bleeding was monitored. Organ spill out after injury was considered as a defect in cuticle integrity. For the survival assay, 100 stage 17 embryos of each genotype were placed alive on apple juice agar plates with yeast (25 embryos per plate). The number of surviving animals was counted throughout larval and pupal development until the hatching of adult flies. To determine overall body size, body circumference in cross sections of first instar wild type and *obst-A* mutant larvae was measured (in pixels) with Image J. The average radius of the sections was determined (in pixels) for calculation of the ideal circumference ( $2\pi r$ ). The actual circumference was compared to the ideal circumference and the discrepancy was statistically analyzed. Calculations were performed for 5 larvae per genotype. For all experiments, significance was calculated using a t-test (Microsoft Excel) and p-values are represented by asterisks: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Error bars show standard error of the mean (SEM) for the integrity test and standard error for the survival assay.

### Quantitative real-time PCR

*obst-A* mutant first instar larvae and wild type larvae from different developmental time points were grown at  $25^\circ\text{C}$ , collected and homogenized in a Precellys homogenizator. RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized using the QuantiTect kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) was performed with a CFX96 cycler (Bio-Rad, München, Germany). For normalization transcript levels of *rp49* (Ribosomal protein L32) were used. qRT-PCRs were carried out of at least five individual biological replicates. Standard control PCR reactions (no-template-control; negative control) were used to rule out contaminations. In addition, primer efficiency (above 95%) as well as potential dimer formation was evaluated. As a control *obst-A* null mutant larvae did not show *obst-A* expression (data not shown), which is consistent with previous findings (19).

Significance was tested using one-way ANOVA (Graph Pad Prism 6) and p-values are indicated by asterisks (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Error bars show the standard error of the mean (SEM). The following primers were used for qRT-PCR analysis: *obst-A*-forward (for) GCAGTGCACAAAGTTCTACG; *obst-A*-reverse (rev) GAACTTGCGGTTGAG-TGGAT; *serp*-for CAAGGCCACCTACTTCGTGT; *serp*-rev ACTGCGATCTCGTGTCTTT; *verm*-for GTGGCTGAAGTCGAAGAAGG; *verm*-rev TTGGTCACGAAGAACACGTC; *knk*-for GGGGCGGATACGTTTTTCTG; *knk*-rev TCCAGAATGTTGGTTTTGCCAT; *ribosomal protein L32* (rp49)-for GCTAAGCTGTGCGACAAATG; rp49-rev GTTCGATCCGTAACCGATGT.

## RESULTS

The chitin-matrix is the main part of the cuticle which is essential for epidermal stability. Barrier integrity is required for survival and shaping of organisms and organs throughout development. Therefore, a number of scaffold proteins and enzymes coordinate chitin-matrix assembly, maturation and degradation at the apical surface of epidermal cells. The Obst-A chitin-binding protein is fundamental for the barrier function of embryonic and larval exoskeletal cuticles. Severe molting defects and lethality at transition to second instar stage in null mutant larvae suggested that Obst-A plays an essential role in cuticle-matrix formation (19).

It has been demonstrated that endogenous Obst-A binds chitin (19). Obst-A protein sequence analysis predicted three subsequent type 2 chitin-binding domains (CBDs) (15). The stereotypic pattern of the three Obst-A CBDs suggested a function in chitin assembly in epithelial organs which require tight and strong cuticles (15). We investigated whether Obst-A is involved in modulating the epidermal chitin-matrix structure. This was addressed by analyzing the overall shape and the ultrastructure of late first instar larval cuticle. The wild type chitin-matrix, specifically marked by the chitin-binding probe (Cbp), was evenly formed at the epidermal apical cell surface. Cross sections revealed in 97% of late first instar wild type larvae ( $n = 27$ ) that epidermis and attached

cuticle straightly lined a rounded body (Fig. 1A). In contrast, 54% of late first instar *obst-A* mutant larvae ( $n=24$ ) showed an extremely wrinkled and massively deformed body shape in cross sections (Fig. 1A). This phenotype is new and unique among known chitin-regulators. In addition, nearly one third (29%) of the larvae revealed cuticle detachment from epidermal cells (Fig. 1B), while wild type was normal. In order to study body shape the relationship between wrinkled cuticle appearance and body size was investigated in wild type and *obst-A* mutants (each  $n=5$ ). We measured the actual circumference of first instar cross sections and the average radius for calculating the ideal circumference. In wild type larvae, the actual cuticle circumference was increased by 9% compared to the ideal circumference due to only few protrusions and notches. In *obst-A* mutant larvae, the size of the buckling cuticle was significantly increased (\*,  $p < 0.05$ ), with a discrepancy to the ideal circumference of 48%. These findings show that normal body appearance depends on Obst-A function during larval development.

The well-defined epidermal cuticle morphology can be visualized in ultrastructure analysis and comprises the outermost envelope, the epicuticle and the inner chitin-rich procuticle. During larval growth, the procuticle develops a highly organized architecture of condensed chitin-fibrils which form an increasing number of regularly stratified chitin-lamellae observed in second instar larvae (Fig. 2A). Importantly, the epidermis contains a distinct and less electron condensed layer between the apical cell surface and the chitin-rich procuticle (Fig. 2A). This defined layer has been previously described as the assembly zone (az) (27) which is synonymous to adhesion zone (28) and deposition zone (14). In the process of cuticle formation, the assembly zone is a stable and permeable matrix, passed by newly synthesized chitin components, but its function in cuticle formation is not yet understood (14,27,28). In contrast to the wild type, the less electron condensed assembly zone was not detectable in *obst-A* mutant larvae. Instead, the procuticle-like structure filled the entire region between epicuticle and epidermal cell surface (Fig. 2A). To further distinguish the procuticle from the



assembly zone, we performed immunogold-labeling using wheat germ agglutinin (WGA), which possesses a strong affinity to oligomers and polymers of N-acetylglucosamine (26). In ultrastructure studies of wild type larvae WGA-gold conjugates were enriched in the compact chitin-rich procuticle, but not within the assembly zone (Fig. 2B,C). In *obst-A* mutants WGA appeared evenly distributed throughout the entire chitin-matrix (Fig. 2B,C). Our findings show that a normal assembly zone between apical cell surface and procuticle was strongly reduced or even absent in *obst-A* mutant epidermis.

We next considered whether Obst-A is involved in epidermal chitin-matrix assembly. In wild type second instar larvae ultrastructure analysis revealed that the chitin-matrix becomes highly organized into multiple chitin-layers (Fig. 2A). In contrast, normal chitin assembly was impaired in *obst-A* mutants resulting in a defective procuticle (Fig. 2A). Chitin-matrix defects would further explain the detached cuticle and wrinkled body shape due to loss of cuticle robustness in *obst-A* mutants (Fig. 1A,B, Fig. 2A). These observations provide evidence that Obst-A is involved in epidermal cuticle formation.

Given that Obst-A is required in the epidermal cuticle assembly zone, we examined its expression by epidermal cells. We performed immunofluorescent labeling studies in the embryonic and larval epidermis by using an antibody that specifically recognizes Obst-A (19). In late wild type embryos epidermal Obst-A protein was apically enriched at the extracellular surface and only little intracellular staining was detected (Fig. 3A), suggesting that Obst-A is secreted by the epidermal cells. This is consistent with the findings that late embryos start to secrete cuticle components before larval hatching (14). For a number of matrix proteins apical deposition depends on septate junction function (11). The claudin-like Megatrachea (Mega) is a core component of septate junctions forming at the lateral membrane in many epithelia including the epidermis (20). Disrupting septate junctions in *mega* mutant embryos led to intracellular enrichment and extracellular reduction of Obst-A in epidermal

cells (Fig. 3B). The *Drosophila* deacetylases, Serp and Verm, are required for late embryonic epidermal cuticle formation. In contrast to *mega*, *serp,verm* double mutant embryos showed normal secretion of epidermal Obst-A towards the developing cuticle (Fig. 3C).

Cuticle secretion is continued during the early first instar larval stage. Co-staining analysis on whole mount first instar larvae with the cell membrane marker  $\alpha$ -Spectrin and the apical surface marker WGA (19,22) revealed a strong overlap of extracellular Obst-A and WGA staining in confocal Z-stack section, orthogonal cross section projections and three dimensional projections (Fig. 3D-D''). Approximately ten hours after hatching larvae undergo apolysis, the separation of epidermal cells from the cuticle, which is followed by the secretion of second instar cuticle (14). In such mid first instar larvae, Obst-A was enriched at the apical cell surface where the assembly zone is expected. Additionally, little Obst-A staining was observed at outer regions of the chitin-matrix (Fig. 3E-E''). It was shown that loss of *obst-A* caused larval lethality at the transition from first to second instar stage (19). Our findings suggest that Obst-A is present during larval transition in both, the outer and therefore first instar cuticle and the newly synthesized second instar cuticle at the cell surface (Fig. 3F-F'').

We further investigated Obst-A localization by TEM ultrastructure analysis combined with immunogold-labeling. In second instar larvae Obst-A was predominantly localized in the assembly zone at the apical cell surface (Fig. 3G). A more detailed study showed that Obst-A staining overlaps with the newly deposited chitin-matrix (Fig. 3H,I). We did not detect Obst-A labeling in *obst-A* null mutant larvae (Fig. 3J). These findings provide evidence that Obst-A is enriched within the assembly zone at the apical epidermal cell surface.

The assembly zone, as the first aECM layer at the apical cell surface, is located between the epidermal cells and its secretory product, the procuticle (27). At the assembly zone chitin-fibrils arise and are most probably organized into the characteristic lamellae of the arthropod procuticle (14,29). As the chitin-matrix requires

renewal during larval molting, we analyzed essential modulators for chitin maturation and protection. It was shown that newly synthesized chitin is protected by the conserved GPI-anchored Knickkopf (Knk) protein for organization of the body wall cuticle in *Tribolium* and for cuticle texture in *Drosophila* trachea and epidermis (4,24,25). In wild type first instar larvae Knk was found in the cuticle matrix, showing strong colocalization with chitin. In late *obst-A* mutant first instar larvae, the Knk protein showed normal colocalization with chitin, but Knk levels appeared upregulated (Fig. 4A-B'). Therefore, relative *knk* expression levels were tested in wild type larvae and compared with *obst-A* mutants by quantitative real-time PCR (qRT-PCR). Approximately 22 hours after larval hatching first instar larvae undergo transition to second instar stage (ecdysis). The first instar stage *knk* levels strongly increased when wild type larvae reached ecdysis and started to molt (Fig. 4C). In contrast, in *obst-A* mutants *knk* levels did not increase for ecdysis 22 hours after hatching. Instead, *knk* became unusually upregulated 26 hours after hatching (Fig. 4C). This late transcript upregulation is consistent with enriched Knk staining (Fig. 4B), suggesting that the dying *obst-A* mutants could try to improve cuticle protection.

Serp as well as the related Vermiform (Verm) both are conserved deacetylase-domain proteins required to improve chitin-matrix maturation, stability and structural durability in embryos (10,11). Immunofluorescent stainings of first instar wild type larvae showed Serp enrichment at the apical cell surface (Figs. 4D,D'). In contrast, loss of *obst-A* resulted in Serp aggregate-like clusters, which appeared mislocalized within the entire chitin-matrix (Fig. 4E-E'). Also, Verm was enriched in the first instar stage at the apical cell surface, but was mislocalized throughout the whole chitin-matrix in *obst-A* mutants (Fig. 4G-H'). Additionally, qRT-PCR analysis of the relative expression levels showed declined *serp* and *verm* expression in *obst-A* mutants specifically 22 hours after larval hatching, when wild type larvae usually undergo ecdysis (Fig. 4F,I). These data indicate that *Obst-A* is involved in Knk, Serp and Verm localization and their function at the epidermal

cuticle.

Larvae need to establish chitin-matrices that withstand mechanical forces. Indeed, pricking of living wild type first instar larvae with a thin glass needle at the posterior lateral epidermis led to small wounds and little bleeding, but was not lethal. Thus, in wild type larvae cuticle barrier integrity is not severely compromised (19). In contrast, large wounds appeared in pricked *obst-A* mutant first instar larvae resulting in severe organ spill out, massive loss of hemolymph and immediate lethality after injury (19). Similar phenotypes of defective cuticle integrity were summarized for UAS-RNAi (interference)-mediated *knk*, *serp* and *verm* knockdown larvae (Fig. 5A), when expressed in the epidermis with the help of the 69BGal4 driver (30,31). It is of note that the knockdown efficiency of those ranged between 88 and 93% (data not shown) and resulted in larval lethality (Fig. 5B). Furthermore, *obst-A* mutant-like cuticle integrity defects were also found in transheterozygous first instar larvae carrying a single mutant allele of *obst-A* and *knk* (*obst-A/+; knk/+*) as well as in transheterozygous larvae of *obst-A* and *serp,verm* (*obst-A/+; serp,verm/+*) (Fig. 5A). These observations further support genetic interaction of *obst-A* with *knk* and with *serp* and *verm* to provide epidermal cuticle integrity.

First instar larvae undergo transition to the second instar stage. Only few hours after larval ecdysis newly secreted second instar chitin-matrix differentiates into the compact procuticle. The process of chitin-matrix reorganization is repeated about 48 hours after larval hatching for the ecdysis of second into third instar larvae (14). The study of relative expression levels showed strong upregulation of *obst-A*, *verm*, *serp* and *knk* during ecdysis into second and third instar. In addition, basic expression levels at intermolt increased from first to third larval stage (Fig. 6A-C). In summary our studies indicate that *Obst-A*, Knk, Serp and Verm could be involved in cuticle assembly and disassembly for larval molting.

After molting, third instar larvae produce almost one chitin-matrix lamella per hour. They dramatically increase the number of chitin-lamellae up to fifty or even more (14) during a

very short time period. A role of Obst-A, Knk, Serp and Verm was tested in regulating such massive cuticle formation. Relative expression showed strong upregulation of *obst-A*, *knk*, *serp* and *verm* at ecdysis from second to third instar. During third instar expression levels of the genes decreased, but remained at high levels when compared to earlier larval stages (Fig. 6A-C).

In immunofluorescent studies WGA was used to detect the apical cell surfaces in *Drosophila* embryos and larvae (19,21,23). Similar to those studies, confocal analysis of third instar epidermis revealed WGA accumulation at the apical cell surface and further labelling of the chitin-matrix (Fig. 6D-I). Co-labeling experiments showed co-enrichment of Obst-A with WGA at the apical cell surface (Fig. 6E). The chitin staining appeared in stratified horizontal lines, probably reflecting the lamellar procuticle morphology. Confocal Z-stacks and orthogonal projections showed that Obst-A is additionally localized in a dotted pattern along those lamellae-like chitin structures (Fig. 6F). In contrast to wild type, in the epidermal cuticle of *serp*, *verm* and *knk* knockdown third instar larvae, extracellular Obst-A was reduced (Fig. 6G-I). In addition, those knockdown mutants revealed a deformed chitin-matrix, though variable in severity (Fig. 6G-I). Collectively, these findings indicate that Obst-A is enriched at the epidermal apical surface and in addition within the outer chitin-matrix in third instar larvae. Furthermore, Obst-A localization depends on *serp*, *verm* and *knk* function.

Analogous to Obst-A, we studied Serp, Verm and Knk localization in the growing third instar cuticle-matrix. Co-labeling studies with WGA detected Serp, Verm and little Knk enrichment at the apical cell surface. While Serp and Verm stainings were weak within the stratified chitin-matrix (Fig. 7A,B), Knk appeared rather distributed towards the chitinous cuticle (Fig. 7C). As shown above, *knk* knockdown larvae resulted in a deformed chitin-matrix. Consequently, not only Obst-A (Fig. 6I), but also Serp and Verm were reduced in the outer chitinous cuticle (Fig. 7D,E). In consistence with the well-described *Tribolium* Knk cuticle protection function (4), our data provide evidence that also *Drosophila* Knk is strongly

required for the newly synthesized chitin-matrix within the lamellate procuticle. Moreover, genetic analysis showed severe cuticle defects in transheterozygous *obst-A* and *knk* (*obst-A/+; knk/+*) mutant third instar larvae. About half of the mutant larvae failed to complete molting and possessed the *obst-A* mutant like wrinkled epidermal cuticle (Fig. 7F). In addition, extracellular Serp and Verm stainings were declined and the chitin matrix was deformed (Fig. 7G,H).

Obst-A, Serp and Verm accumulate at the apical surface, where chitin is modified and packaged into the lamellae. In *obst-A* null mutant first instar larvae apical enrichment of Serp and Verm was not detectable. Similar phenotypes were observed in transheterozygous *obst-A* and *serp,verm* (*obst-A/+; serp,verm/+*) mutant third instar larvae. Confocal sections and orthogonal projections showed a deformed epidermal chitin-matrix and reduced stainings of Knk (Fig. 7I-L). In summary, our data suggest that Obst-A, Serp, Verm as well as Knk genetically interact in packaging and protecting the chitin-matrix of the epidermal cuticle.

## DISCUSSION

Molting, wounding and other types of cuticle disruption require complex actions of enzymes and scaffold proteins that form new and remodel existing cuticles throughout development (4,32,33). To some extent chitin synthases and chitinolytic enzymes have been studied in the past (1-7), but little is known about underlying mechanisms that control assembly, stability and integrity of newly synthesized cuticles. The assembly zone is the first apical extracellular area where all cuticle components are deposited and the chitin-matrix is packaged into the highly ordered procuticle. It has been discussed that the assembly zone is a permeable matrix for components that process into the cuticle (27). Indeed, cuticle proteins and putative enzymes were identified to be part of the assembly zone (27), however, they have not been molecularly characterized.

Our data demonstrate that Obst-A plays a key role in organizing the chitin-matrix. Normal chitin levels in *obst-A* null mutants exclude a



role in chitin synthesis. Given that Obst-A is required for exoskeletal function at the epidermis (Figs. 1,2) (19), we investigated the extracellular region where Obst-A organizes the chitin-matrix. Indeed, confocal, ultrastructure and mutant analysis identify Obst-A as a chitin-binding protein essential for assembly zone formation. Moreover, Obst-A and its partner proteins are required for larval cuticle stability (Fig. 5A) (19). In addition, our observations about *obst-A* expression pattern are in line with recent data showing that loss of *obst-A* results in severe molting defects and lethality shortly after ecdysis to second instar larval stage (19). Collectively, Obst-A is an essential regulator at the apical cell surfaces coordinating chitin-matrix formation and thereby promoting epidermal cuticle integrity.

Cuticle defects in *obst-A* mutants could implicate impaired protection of newly synthesized cuticle. In the beetle *Tribolium castaneum* the Knk protein is required for protection of newly synthesized chitin-matrix (4). In the embryonic tracheal matrix Obst-A maintains extracellular Knk localization, which prevents premature degradation of the cuticle during tracheal tube size control (19). Few hours later at the end of embryogenesis dispensable intraluminal chitin-matrix becomes cleared by clathrin-mediated endocytosis (21,22) resulting in a rather thin apical chitin-rich cuticle (23). Here we found that epidermal extracellular Knk colocalization with chitin was not affected in *obst-A* mutants and conversely weak apical Obst-A enrichment was found in *knk* knockdown larvae. However, *knk* gene and protein levels appeared reduced in the *obst-A* mutants at a time when wild type larvae start to molt. This suggests that chitin-matrix protection must be affected. Our findings may not exclude other potential protective proteins (32) and yet uncharacterized *Drosophila* Knk-like proteins (33) could depend on Obst-A. However, the wrinkled cuticle specifically observed in *obst-A* null and transheterozygous *obst-A;knk* mutants and the localization of Obst-A and Knk proteins in the outer cuticle leads to the speculative hypothesis that they act in cuticle stability of the newly synthesized and packaged chitin matrix. In summary, our findings might further suggest that aspects of *obst-A* mutant cuticle phenotypes

could be the result from mistimed or ectopic degradation of chitin in the exoskeleton.

The procuticle is capable of extending throughout larval development, while its integrity remains stable. Previous data about Obst-A binding with chitin and our ultrastructure observations suggest that Obst-A could play a role in coordinating chitin-scaffold formation. This would be consistent with gene expression data, protein localization and genetic studies which propose a genetic link of *obst-A* with *serp* and *verm* in organizing maturation of the epidermal cuticle throughout larval development. Our data further suggest that Obst-A proteins may recruit chitin-fibrils at the apical cell surface to organize their packaging and maturation into a more compact procuticle. In addition, we observed Obst-A scattering along the stratified chitin-lamellae in third instar larval cuticle. Therefore, it is also possible that Obst-A is involved in providing stability of the outer epidermal cuticle.

Previously it was described that the zona pellucida protein Piopio provides a structural network in trachea (34) that may link the apical epithelial cell surface with the overlaying aECM at the epidermis (28). In addition, the  $\delta$ -Aminolevulinate synthase (Alas), expressed in the hepatocyte-like oenocytes, is involved in formation of a dityrosine network at the apical cell surface to resist hydrostatic pressure of the hemolymph and to prevent dehydration (35). Local detachment between cell surface and cuticle observed in *piopio* and *alas* mutants (28,35) was also identified in *obst-A* null mutants (Fig. 1B). Interestingly, cuticle detachment was found in *obst-A* mutants, *serp* knockdown larvae and in the transheterozygous *obst-A serp,verm* mutants. Due to defective cuticle structure in *obst-A* mutants we speculate that cuticle formation at the apical cell surface, likely within the assembly zone, affects essential aspects of cell adhesion to the aECM (28).

Our data provide evidence that Obst-A is required for the assembly zone formation. Whether Obst-A may act in the elongation or ordering of chitin-filaments or whether it prevents premature chitin-fibril assembly, remains elusive. However, our data propose that

Obst-A provides a well-structured chitin-scaffold for deacetylation enzymes to mature and improve aECM stability and integrity. Furthermore, genetic studies suggest that Obst-A is linked to Knk-mediated protection of the newly synthesized cuticle (Fig. 8). All insects establish compact and structured exoskeletal cuticles at their outermost body parts (4,29,32,33,36,37). Our data argue that structural similarities of the body wall cuticle and molecular conservation of involved proteins (8,9,15-19,38-40) point toward a highly conserved mechanism of procuticle formation among chitinous invertebrates.

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## FOOTNOTES

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<sup>2</sup>The abbreviations used are: aECM, apical extracellular matrix; CBD, chitin-binding domain; Cbp, chitin-binding probe; ep, epidermis; knk, knickkopf; mega, megatrachea; obst-A, obstructor-A; pro, procuticle; serp, serpentine; ts, tracheal system; verm, vermiform; WGA, wheat germ agglutinin

## FIGURE LEGENDS

### FIGURE 1

#### Obst-A is required for larval body shape control and epidermal cuticle integrity

(A,B) Confocal and bright field (bf) images show larval cross sections. Chitin is visualized by Cbp (green) and WGA (grey, B) marks chitin and the apical cell surfaces. (A) In 97% (n=27) of wild type first instar larvae the cuticle (green) is evenly attached to the epidermal cells. In 54% (n=24) of *obst-A* mutant first instar larvae the epidermal cuticle is wrinkled and the body circumference is increased. Images are generated by confocal sections and bright field microscopy. Upper panel shows cross sections of the larvae (arrows point to the epidermis) and lower panel magnifications of the epidermal cuticle. (B) In 97% (n=27) of wild type larvae the cuticle is tightly attached to epidermal cells and marked by WGA. In 29% (n=24) of *obst-A* mutant larvae, the cuticle was detached (arrows) from the underlying epidermal cells (arrows). Scale bars represent 50  $\mu$ m or 5  $\mu$ m, respectively.

### FIGURE 2

#### Obst-A organizes chitin-matrix structure at the assembly zone

(A) The wild type epidermal cuticle (left panel) of second instar larvae is stratified into the outermost envelope, the epicuticle (epi) and the prominent inner procuticle (pro). The procuticle contains increasing number of chitin-lamellae. Arrow points to the procuticle. Red dashes mark borders between chitin-layers of the procuticle and blue dashes point to the apical cell membrane. In wild type larvae, the less electron dense assembly zone (az) is localized between apical cell surface and procuticle. In contrast, the assembly zone is largely diminished in *obst-A* mutant larvae (n=3; right panel). Rudimentary procuticle organization is detectable within the entire chitin-matrix. Magnifications of the procuticle show highly organized chitin-matrix in the wild type but impaired architecture in *obst-A* mutants. A wrinkled structure of the epidermal aECM is found in *obst-A* mutants. Scale bars in magnifications represent 100 nm.

(B,C) Overview (B) and magnifications (C) of the ultrastructure show immunogold-labelled WGA that recognizes chitin. WGA labeling is strongly enriched in the procuticle but less in the assembly zone of wild type second instar larvae. In contrast, in *obst-A* mutant larval epidermis WGA is evenly distributed in the chitin-matrix and a distinct assembly zone is not detected. Procuticle (pro), assembly zone (az). Blue dashes label the apical cell surface and red dashes indicate where the procuticle starts. Scale bars represent 1  $\mu$ m or 500 nm, respectively.

### FIGURE 3

#### Epidermal Obst-A is localized within the cuticle

(A-C) Confocal images show epidermal cells of stage 17 embryos labeled with anti-Obst-A (red), the

membrane marker anti- $\alpha$ -Spectrin (Spec, blue) and WGA (green) marking the apical cell surface (green). Single Obst-A channel is presented in grey. Scale bars indicate 5  $\mu$ m. **(A)** In stage 17 wild type embryos, Obst-A is enriched (arrow) at the apical cell surface, which is marked by WGA. Only weak intracellular Obst-A distribution is found within epidermal cells. Spec marks plasma membranes of epidermal cells. **(B)** In stage 17 *mega* mutant embryos, Obst-A remains in epidermal cells (arrow), and extracellular Obst-A staining was reduced. **(C)** In stage 17 *serp, verm* mutants, Obst-A was normally enriched at the apical cell surface (arrow).

**(D-D'')** Confocal analysis of whole mount staining of a wild type first instar larva labeled with anti-Obst-A (red), WGA (blue) and anti- $\alpha$ -Spectrin (Spec, green). Confocal scan (D), three dimensional (D') and orthogonal projections (op) in D'' of the epidermis demonstrate extracellular Obst-A overlap (arrows) with the apical cell surface marker WGA (D'' lower panel) but not with  $\alpha$ -Spectrin (D'' middle panel).

**(E,F)** Confocal images of larval cross-sections, labelled with Obst-A (red), Cbp (chitin, green) and WGA (blue) and bright field (bf) microscopy. **(E-F''')** In mid (E-E''') and late (F-F''') first instar larval epidermis both newly deposited cuticle and old first instar cuticle are detected (14). Obst-A is localized at the apical cell surface together with newly deposited chitin (arrows) and at outer regions (yellow and blue arrowheads), probably representing the old first instar cuticle. Obst-A staining overlaps with WGA (E'', F'') and chitin (Cbp, E''', F'''). Analysis of first and second instar larvae  $n \geq 20$ . Scale bars represent 5  $\mu$ m.

**(G-J)** Immunogold-labeling of Obst-A in second instar wild type (G-I) and *obst-A* mutant epidermis (J). **(G)** In second instar wild type larval epidermis, immunogold-labeling of Obst-A (arrows) is enriched at the apical cell surface but underneath the procuticle (pro). The inset shows a high magnification. Blue dashes indicate the apical cell surface and red dashes the start of the procuticle. Apical cell surface is at the bottom in all images. Assembly zone (az). Scale bars represent 1  $\mu$ m. **(H,I)** Single or even several immunogold particles labeling Obst-A (arrows) overlap with chitin-fibrils within the assembly zone. Scale bars represent 500 nm. **(J)** In *obst-A* null mutant larvae no immunogold particles were observed at the chitin-matrix. Blue dashes indicate the apical cell surface. Procuticle (pro), Scale bars represent 1  $\mu$ m.

## FIGURE 4

### Epidermal localization and expression of the chitin protector Knk and the chitin deacetylases Serp and Verm

Upper and middle panels show confocal images of first instar larval cross-sections, labelled with anti-Knk (red), anti-Serp (red), anti-Verm (red) and Cbp (chitin, green). The lower panel provides qRT-PCR analysis of relative *knk* (C), *serp* (F) and *verm* (I) expression in wild type and *obst-A* null mutant larvae.

**(A-B')** Knk localization in wild type (A-A') and *obst-A* mutant (B-B') epidermis. In late first instar larval epidermis, Knk is distributed in the chitin-matrix (arrow in A) where it colocalizes with the chitin marker Cbp (A,A'). In late first instar *obst-A* mutant epidermis, Knk staining appeared slightly enriched, but revealed a wild type-like distribution (B,B').

**(D-E')** In wild type mid first instar epidermis, Serp staining is enriched at the apical cell surface (arrows in D). Serp staining is not distributed throughout the entire chitin-matrix, as it only partially overlaps with chitin as detected by Cbp (D'). In first instar *obst-A* mutant epidermis, Serp staining is mislocalized and is found throughout the chitin-matrix without enrichment at the apical cell surface (E,E'). **(G-H')** Verm localization in wild type (G-G') and *obst-A* mutant (H-H') epidermis. Verm staining resembles Serp pattern. Verm is enriched in the newly deposited chitin-matrix (arrows in G) where it partially overlaps with chitin (G'). In first instar *obst-A* mutant epidermis, Verm is mislocalized within the chitin-matrix. Analysis of wild type and *obst-A* mutant larvae  $n \geq 20$ . Scale bars represent 5  $\mu$ m.

**(C,F,I)** qRT-PCR analysis of relative expression in wild type and *obst-A* null mutant larvae shows a significant upregulation of *knk*, *serp* and *verm* levels 22 h after larval hatching, which corresponds to ecdysis from first to second instar larval stage. In *obst-A* mutants, however, *knk*, *serp* and *verm* expression is not upregulated at 22 h after larval hatching. *knk* levels are significantly enriched at 26 h after larval hatching, shortly before *obst-A* mutants start to die. Significance (one-way ANOVA) is compared to values 0 hours after hatching of wild type larvae and indicated by asterisks and standard error of the mean is represented by bars. p-values are represented by asterisks: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**FIGURE 5*****knk*, *serp* and *verm* are required for epidermal cuticle integrity and larval survival**

(A) Cuticle integrity test was performed by pricking first instar larvae according to (19). 84 % of wild type larvae (n=94) survived pricking without severe wounding, whereas 93 % of *obst-A* mutant larvae (n=46) showed severe epidermal disruption, which caused organ spill and immediate lethality. A large number of transheterozygous larvae (*obst-A/+;knk/+* (n=60) and *obst-A/+;serp,verm/+* (n=65)) showed *obst-A* mutant-like cuticle integrity defects. Similar cuticle integrity defects were observed in *knk* (48 %, n=103), *serp* (52 %, n=66) and *verm* (59 %, n=58) knockdown larvae. The p-values are represented by asterisks: \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

(B) Survival tests revealed larval lethality for *knk*, *serp*, and *verm* knockdown mutants, but not for wild type. The test was repeated four times with n=25 larvae of each genotype. Less than 1% of *verm* knockdown larvae reach the third instar stage. Error bars represent the standard error and p-values are represented by asterisks. Note that the knockdown efficiency for all RNAi lines as determined by qRT-PCR was approximately 90 % (data not shown).

**FIGURE 6*****Knk*, *Serp* and *Verm* are involved in larval *Obst-A* expression and its localization within the cuticle**

(A-C) qRT-PCR analysis shows relative expression levels of *obst-A* (A), *serp* and *verm* (B) and *knk* (C) throughout larval development. *obst-A*, *serp*, *verm* and *knk* expression levels are upregulated during ecdysis to second and third instar stages. Significance (one-way ANOVA) is compared to values 0 hours after larval hatching and indicated by asterisks and standard error of the mean (SEM) is represented by bars. p-values are represented by asterisks: \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

(D-I) Confocal images of third instar larval cross-sections, labelled with *Obst-A* (red), *Cbp* (chitin, green) and WGA (blue). Orthogonal projections of Z-stacks are depicted in small images. (D,E) In wild type third instar larvae, chitin (*Cbp*, green) and the apical cell surface marker WGA (blue) colocalize with *Obst-A* (red) staining at the epidermal apical cell surface (white arrows). *Obst-A* (red) alone is depicted at the right side. *Obst-A* is enriched at the apical cell surface and in addition lower levels were detected intracellularly (white arrowhead) and in the chitin-rich procuticle (yellow arrowhead). (F) The third instar procuticle consists of several distinct chitin lamellae, as indicated by the stratified chitin staining. *Obst-A* colocalizes with the lamella-like chitin (arrowhead, left image) and with WGA (blue) detected in the chitin-matrix. Orthogonal projections are presented in small images and *Obst-A* alone in the central image. (G-I) *serp*, *verm*, *knk* knockdown larvae (n≥18) show defective chitin-matrix organization and extracellular *Obst-A* staining appeared reduced (yellow arrowhead), but *Obst-A* enrichment at the apical cell surface is detected in *serp* and *knk* and partially in *verm* knockdown (arrows). The cuticle is detached in *serp*, but not in *knk* or *verm* knockdown larvae. Orthogonal projections (op). Scale bars represent 5 µm.

**FIGURE 7****Genetic control of epidermal cuticle organization in *Drosophila* larvae**

Confocal images of third instar larval cross-sections, labelled with *Serp* (red, A; D; G), *Verm* (red, B; E; H), *Knk* (red, C; I), *Obst-A* (red, F). Chitin is detected by *Cbp* (green) and the apical cell surface by WGA (blue). Orthogonal (op) projections of Z-stacks are depicted in small images. (A-B) *Serp* and *Verm* are strongly enriched at the apical cell surface (white arrows) overlapping with chitin (*Cbp*, green) and WGA (yellow arrows). Orthogonal projections confirm enrichment at the apical cell surface overlapping with the chitin-matrix.

(C) *Knk* signal is enriched at (white arrow), but not restricted to the apical cell surface. *Knk* is distributed throughout the whole chitinous procuticle (yellow arrows). The 3D projection and the orthogonal projection visualize the distribution of *Knk* within the whole procuticle.

(D,E) The RNAi-mediated knockdown of *knk* shows reduced extracellular *Serp* (D) and *Verm* (E) staining, which, however, appeared still enriched at the apical cell surface (arrows).

(F-H) In transheterozygous *obst-A/+;knk/+* third instar larvae (n=6), chitin-matrix is deformed, cuticle molting is defective (arrows in F and G) and extracellular *Serp* (G) and *Verm* (H) stainings are decreased.

About 50% of such mutant larvae frequently show *obst-A* mutant-like wrinkled epidermal cuticle (arrows in F), but not detached cuticle. The inset highlights the wrinkled newly synthesized cuticle and the outer old cuticle.

**(I-L)** In transheterozygous *obst-A/+;serp,verm/+* third instar larvae (n=14), chitin-matrix organization is defective and the cuticle is frequently detached from the epidermis. These mutants show reduced extracellular Knk (red, I), Serp (red, J), Verm (red, K), and Obst-A (red, L) stainings. Transheterozygous mutants show strong reduction or loss of Obst-A, Serp and Verm accumulation at the apical cell surface. The transheterozygous *obst-A/+;serp,verm/+* mutants show detached (6 of 14), but not wrinkled cuticle. Scale bars represent 5  $\mu$ m and 50  $\mu$ m in F, respectively.

## FIGURE 8

### Obst-A organizes the aECM at the apical cell surface

Schematic model illustrates Obst-A in the cuticle. Our data provide evidence that Obst-A arranges chitin-matrix maturation and protection at the apical cell surface. We propose that Obst-A controls chitin-scaffold formation. Obst-A acts in a common pathway with the deacetylation enzymes Serp and Verm and with the chitin protector Knk to mature, stabilize and protect the aECM within the cuticle.



Figure 1

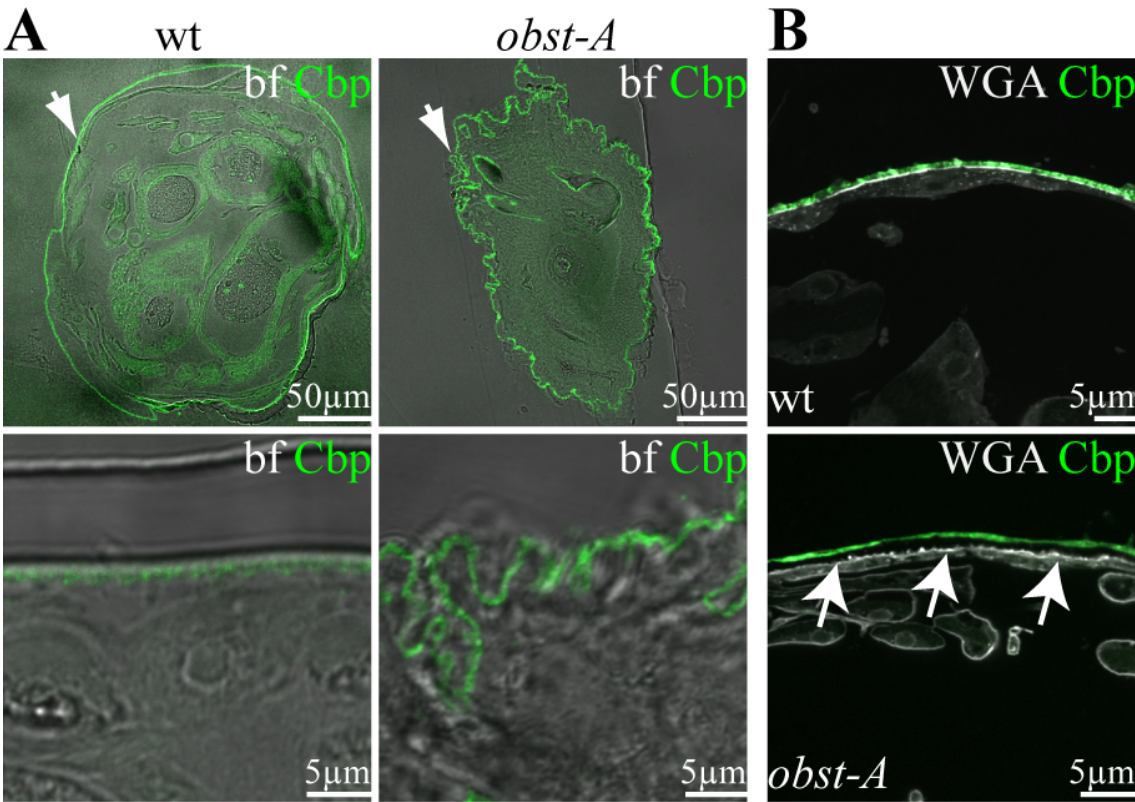
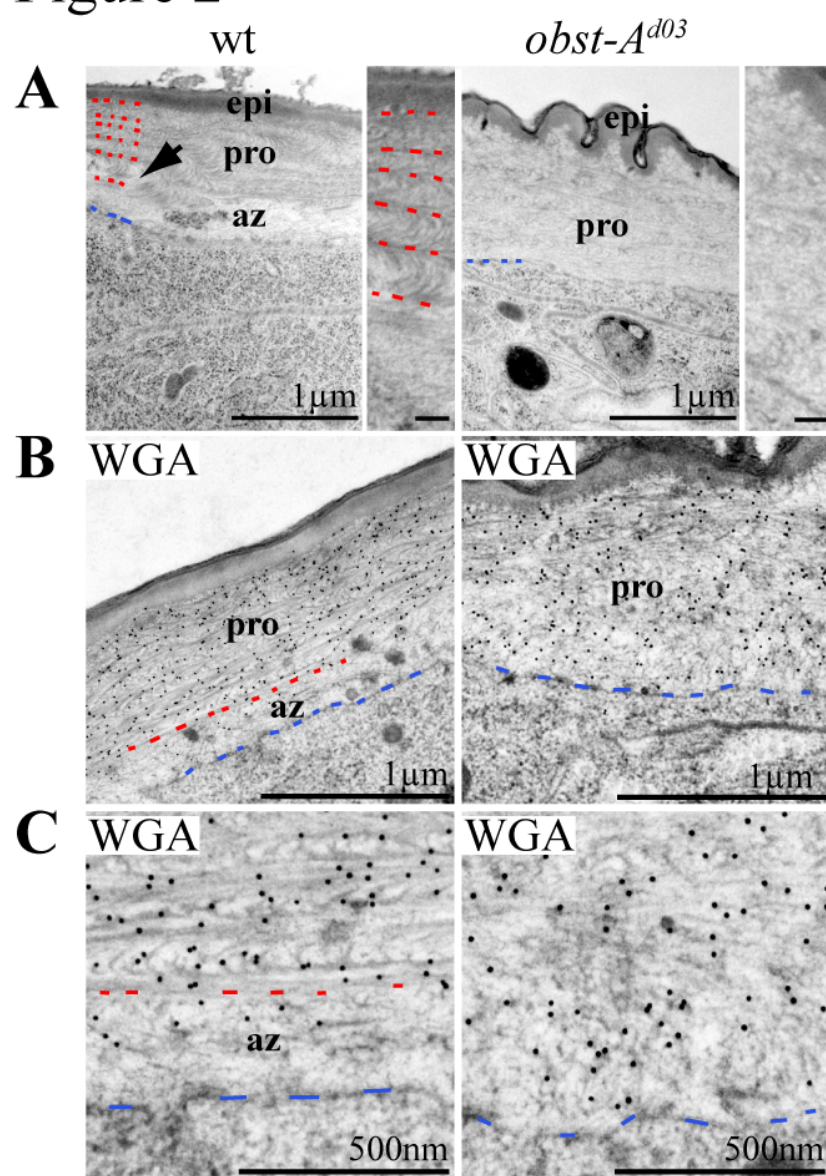
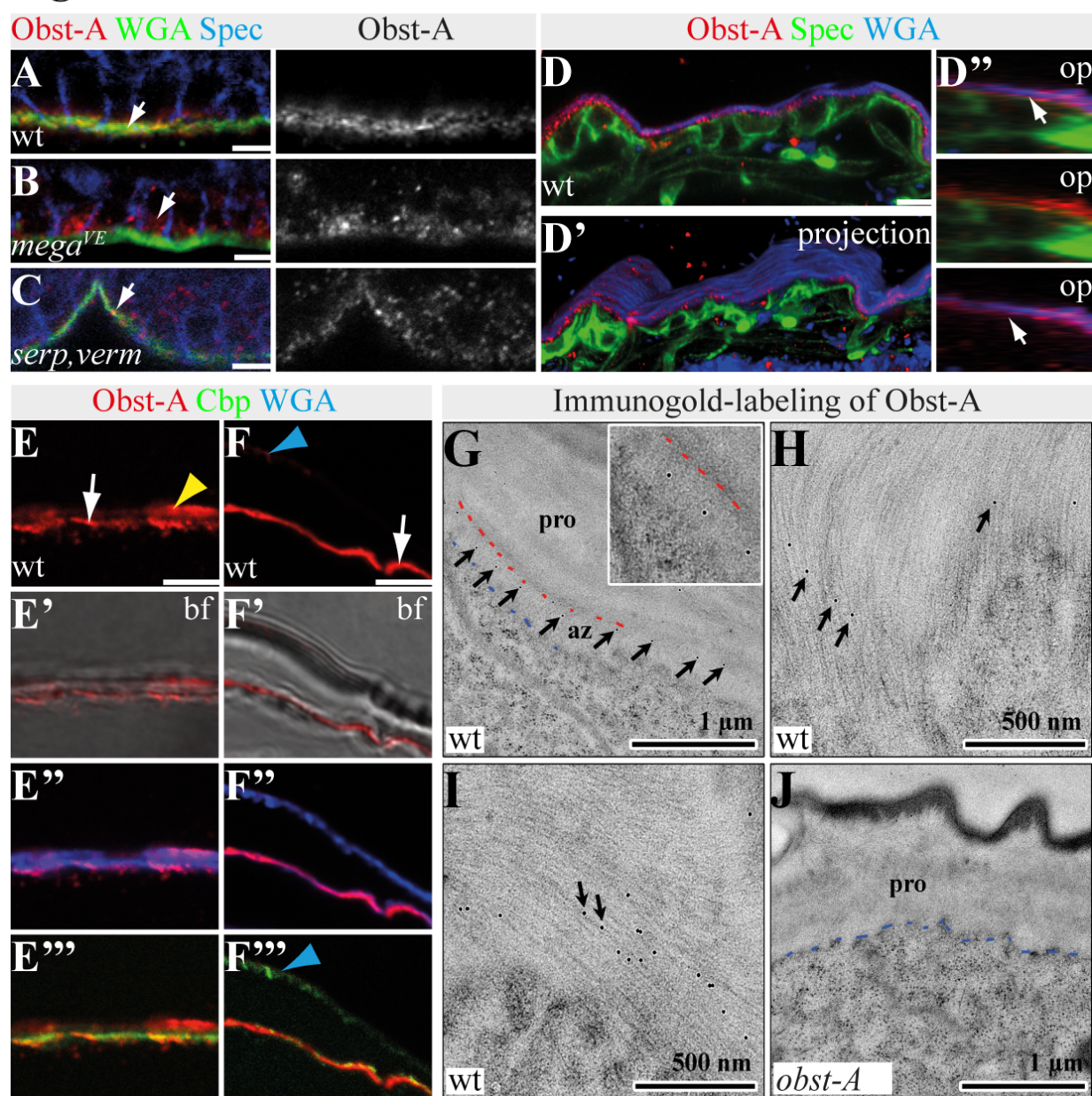


Figure 2

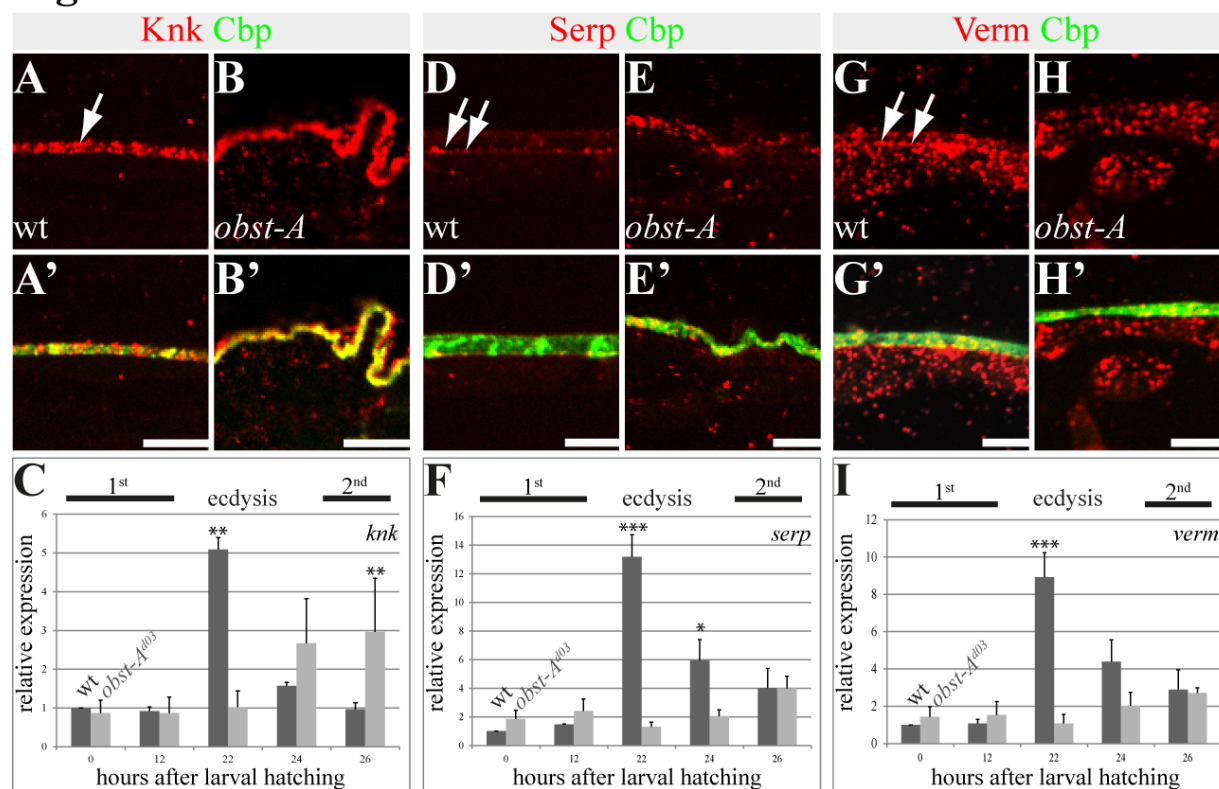




**Figure 3**



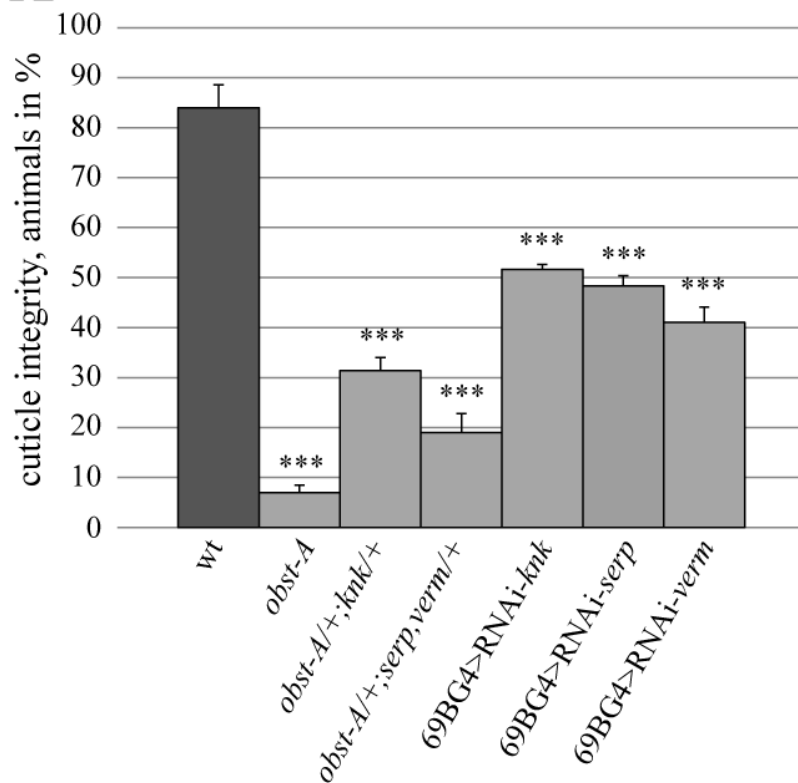
**Figure 4**



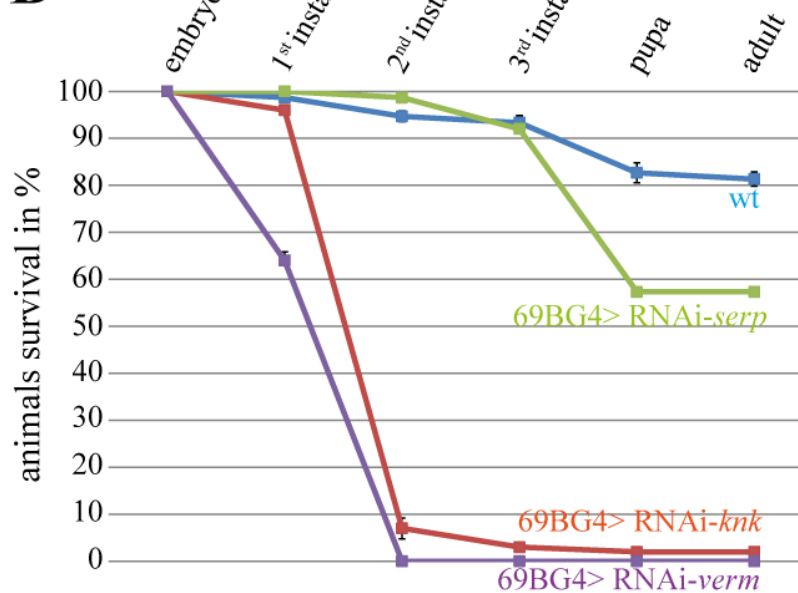


**Figure 5**

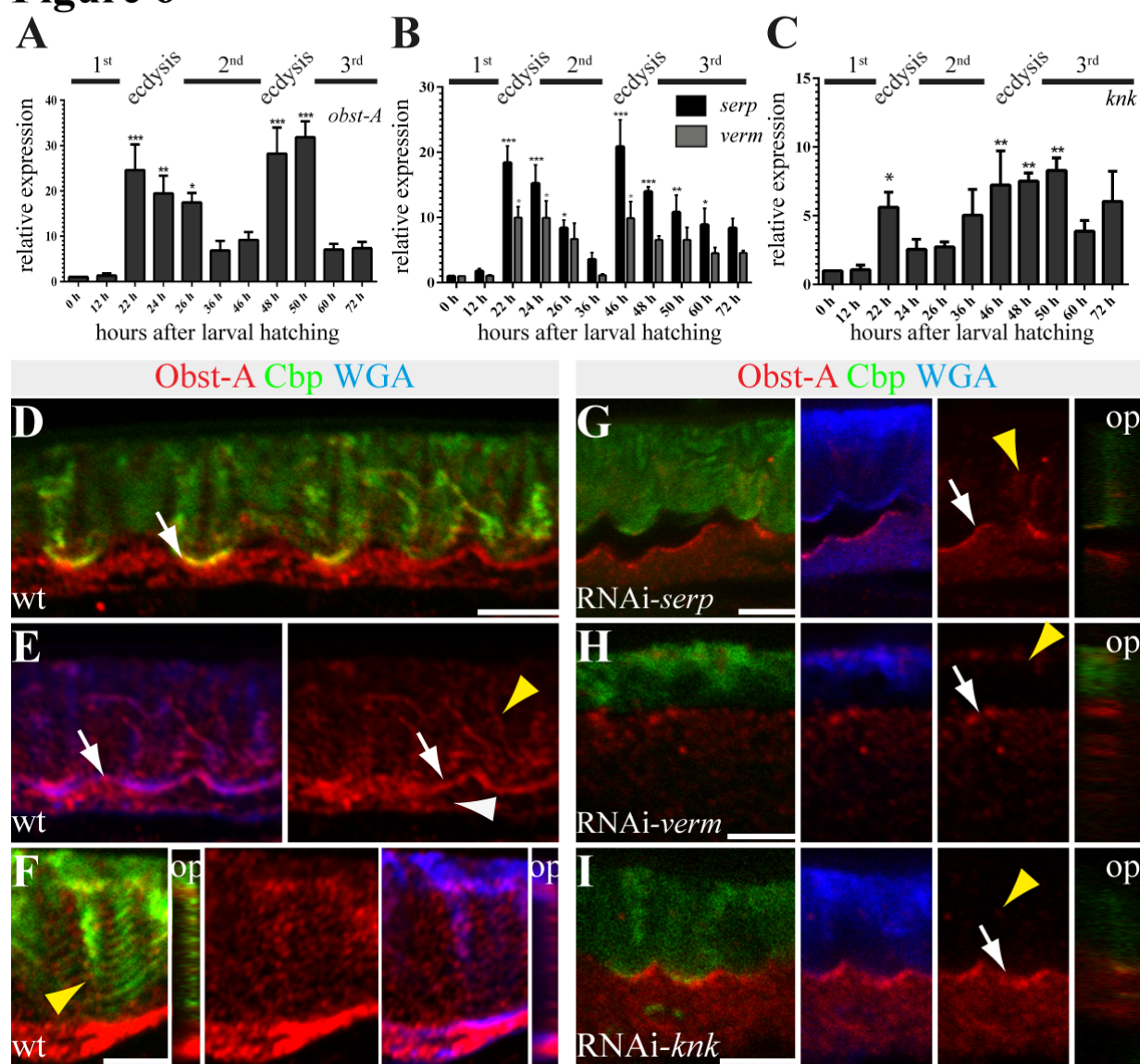
**A**



**B**



**Figure 6**



**Figure 7**

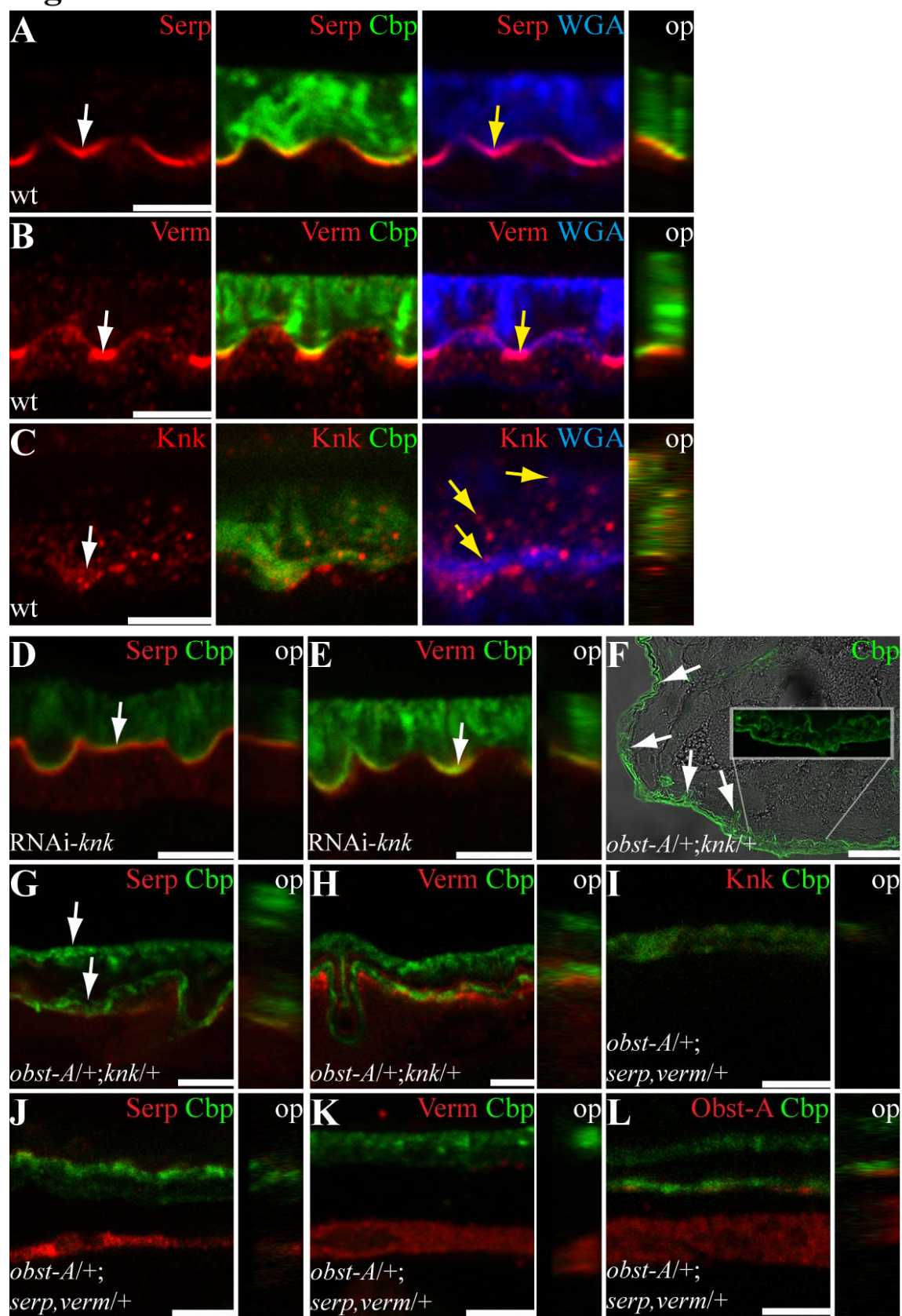
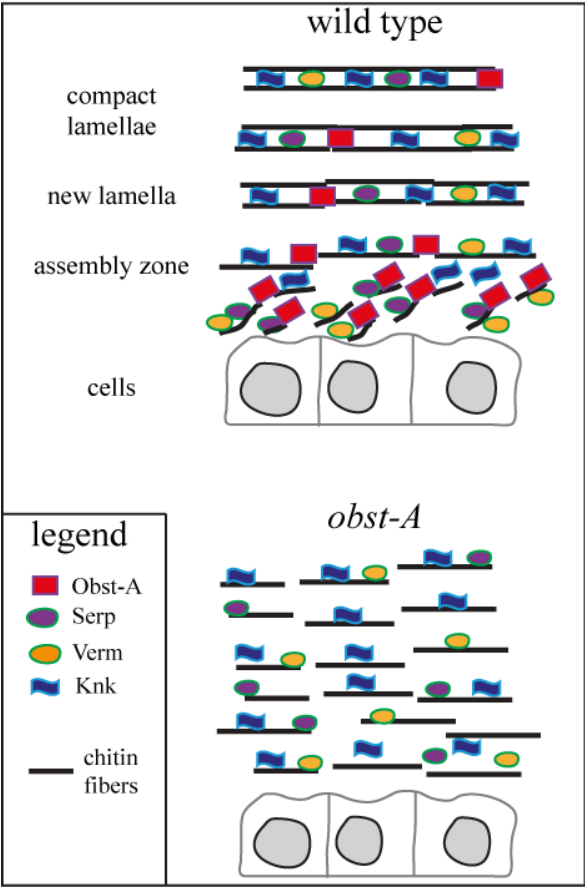


Figure 8





**Developmental Biology:**

**Obstructor-A organizes matrix assembly at the apical cell surface to promote enzymatic cuticle maturation in *Drosophila***

Yanina-Yasmin Pesch, Dietmar Riedel and Matthias Behr

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