---

**Article:**
Sobala, Lukasz F orcid.org/0000-0002-3807-6452, Wang, Ying and Adler, Paul N (2016) ChtVis-Tomato, a genetic reporter for in vivo visualization of chitin deposition in Drosophila. Development (Cambridge, England). pp. 3974-3981. ISSN 1477-9129

https://doi.org/10.1242/dev.126987

---

© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 3638 doi:10.1242/dev.143826

CORRECTION

# Correction: ChtVis-Tomato, a genetic reporter for *in vivo* visualization of chitin deposition in *Drosophila*

**Lukasz F. Sobala, Ying Wang and Paul N. Adler**

There was an error published in Development **142**, 3974-3981.

In the Materials and Methods, it was stated that the pWalium-ChtVis-Tomato plasmid is available from Addgene (72102). The plasmid number was incorrect and should have been 67756.

This error does not affect the conclusions of the paper. The authors apologise to readers for this mistake.

© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

RESEARCH ARTICLE

TECHNIQUES AND RESOURCES

# ChtVis-Tomato, a genetic reporter for *in vivo* visualization of chitin deposition in *Drosophila*

**Lukasz F. Sobala*, Ying Wang and Paul N. Adler‡**

## ABSTRACT

Chitin is a polymer of N-acetylglucosamine that is abundant and widely found in the biological world. It is an important constituent of the cuticular exoskeleton that plays a key role in the insect life cycle. To date, the study of chitin deposition during cuticle formation has been limited by the lack of a method to detect it in living organisms. To overcome this limitation, we have developed ChtVis-Tomato, an *in vivo* reporter for chitin in *Drosophila*. ChtVis-Tomato encodes a fusion protein that contains an apical secretion signal, a chitin-binding domain (CBD), a fluorescent protein and a cleavage site to release it from the plasma membrane. The chitin reporter allowed us to study chitin deposition in time lapse experiments and by using it we have identified unexpected deposits of chitin fibers in *Drosophila* pupae. ChtVis-Tomato should facilitate future studies on chitin in *Drosophila* and other insects.

**KEY WORDS: Chitin reporter, Cuticle, *Drosophila*, *In vivo* imaging**

## INTRODUCTION

Chitin is generally considered to be the second most abundant biomolecule. It is a polymer of N-acetylglucosamine linked by β-1,4 glycosidic bonds and is synthesized by the enzyme chitin synthase, which exists as a transmembrane protein (Merzendorfer and Zimoch, 2003). It is found in a wide variety of organisms including microorganisms such as fungi, protists and algae, arthropods such as insects, crustaceans and arachnids, and in other invertebrates such as sponges, coelenterates, molluscs and nematodes. Recently it has become clear that chitin is also produced in a number of vertebrates (Tang et al., 2015) and that some vertebrate genomes have recognizable chitin synthases (Zakrzewski et al., 2014). Chitin usually functions as a structural component of cell walls or extracellular matrix. In fungi it is often deposited at sites of rapid growth (Molano et al., 1980; Sloat and Pringle, 1978; Teparić and Mrša, 2013) and in insects it is deposited at the apical surface of epithelial cells as part of the cuticular exoskeleton (Merzendorfer, 2013; Merzendorfer and Zimoch, 2003; Moussian, 2013).

From a morphological perspective, the most complex biological structures that contain chitin are the exoskeletons of insects and other arthropods, which are decorated with a wide range of specialized structures including sensory bristles, hairs (trichomes), ridges and various types of sensilla. In addition to chitin, the insect exoskeleton cuticle is composed of cuticle proteins, lipids, minerals and water (Vincent, 2005). It has a complex layered structure and from one body region to another it varies enormously in its physical properties. These differences are presumably a result of differences in the content and arrangement of molecular constituents but for the most part, this has not been well studied. One difficulty is the large number of cuticle proteins encoded by insect genomes. For example, there are ∼150 cuticle protein genes annotated in the *Drosophila* genome and more than 200 in some other insects (Cornman, 2010; Willis, 2010). In contrast, there are only two chitin synthases encoded by insect genomes and only one of these functions in the synthesis of cuticle (Arakane et al., 2005; dos Santos et al., 2015; Gagou et al., 2002; Moussian et al., 2005). Hence, there are advantages to studying chitin and chitin synthase as a starting point for trying to understand the genetic basis for the differences between cuticles.

Several different approaches have been used to localize chitin in cells and tissues. Many investigators have stained fixed or living cells using Calcofluor (Sloat and Pringle, 1978), Congo Red (Michels and Buntzow, 2010) or wheat germ agglutinin (Molano et al., 1980; Tronchin et al., 1981). However, these approaches suffer from a lack of specificity as other carbohydrates can also be recognized by the reagents. They have primarily been used on yeast and fungi and in our experience are not well suited for staining insect cuticle. A more specific staining protocol has used a fluorescently labeled protein that contains a CBD. This has been used successfully in fungi, nematodes and insects (Gangishetti et al., 2009; Nagaraj and Adler, 2012; Taylor et al., 2002; Zhang et al., 2005). There are, however, limitations with this staining approach. It is most often used on fixed material, cannot be used for time lapse experiments and, at least for insect cuticle, the ability to stain chitin is progressively lost as cuticle development proceeds (Adler et al., 2013; Nagaraj and Adler, 2012). Successful staining is also sensitive to the degree of fixation and this sensitivity increases progressively as differentiation proceeds. The basis for these limitations is likely decreased accessibility for the tagged protein because of the tightly packed and cross-linked nature of cuticle. Consistent with this hypothesis is the dramatic decrease in the ability to immunostain cellular constituents through developing cuticle. To overcome these limitations we have developed a genetic *in vivo* chitin reporter for *Drosophila*. The reporter protein consists of amino and carboxy terminal segments of the Dyl protein, which is a ZP domain protein that is secreted apically and is important for cuticle deposition (Adler et al., 2013; Fernandes et al., 2010; Nagaraj and Adler, 2012). Internally, we placed the chitin-binding domain of chitinase A1 from *Bacillus circulans* (Watanabe et al., 1994) and a copy of td-Tomato, an extremely bright red fluorescent protein (Shaner et al., 2004). DNA encoding this fusion protein was subcloned into a UAS vector and transgenic flies generated. This reporter proved to be both effective and sensitive at detecting chitin. It enabled us to carry out time lapse experiments and to detect chitin

Biology Department and Cell Biology Department, University of Virginia, Charlottesville, VA 22904, USA.
*Present address: Technical University of Lodz/York Structural Biology Laboratory, YSBL, Department of Chemistry, Heslington Road, University of York, York YO10 5DD, UK.

‡Author for correspondence (pna@virginia.edu)

**Received 29 May 2015; Accepted 8 September 2015**

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

long after this would be possible by standard staining protocols. We constructed a control gene where the CBD was not included, which we used to compare with ChtVis-Tomato. The expressed control protein failed to stain chitin-containing structures, confirming the specificity of the reporter containing the CBD. We also showed that in extracts of imaginal wing discs, the reporter would bind to chitin beads while the control did not. Using the reporter, we identified an unexpected deposit of chitin fibers located between the apical surface of imaginal epithelial cells and the pupal cuticle. These fibers were observed ~13 h prior to the time we could detect chitin deposition in the adult cuticle and were gradually lost as development proceeded.

## RESULTS

### The ChtVis-Tomato protein is secreted apically and binds to chitin

The reporter protein was designed to mimic several properties of the Dyl protein (Adler et al., 2013). Dyl is thought to be a single-pass transmembrane protein with the amino terminus outside of the cell. A putative furin cleavage site is found before the putative transmembrane domain, and we previously found that the Dyl protein was secreted and not tethered to wing cells (Adler et al., 2013). We hypothesize that the protein was cleaved at the furin site, which allowed it to diffuse away from the synthesizing cell. In at least one ZP domain-containing protein this cleavage is important for protein activation (Jovine et al., 2004). The reporter contains both the Dyl signal sequence to drive the secretion of the amino terminal segment and the furin site to allow release from the membrane (Fig. 1). In between these *dyl*-derived sequences we inserted a CBD from the *Bacillus circulans* chitinase A1 gene (Watanabe et al., 1994), and a copy of the very bright td-Tomato protein (Shaner et al., 2004). To test if the reporter was secreted we expressed both the reporter and GFP using *en-Gal4*, which drives expression in the posterior compartment of imaginal discs and in stripes in embryonic and larval segments. As expected, GFP accumulated only in posterior compartment cells where it is expressed (Fig. 2A). By contrast, we observed td-Tomato fluorescence throughout the space located between the apical surface of wing disc cells and the peripodial membrane (Fig. 2A) as expected for a secreted protein. We also examined the larval body wall and observed fluorescent stripes expressing both GFP and ChtVis-Tomato (Fig. 2B). This seemed likely to be a result of the reporter binding to larval body wall chitin and hence not diffusing away from the synthesizing cell. As a further test of the specificity of the reporter we compared the fluorescence pattern in the hypoderm of *en-Gal4 UAS-GFP/UAS-ChtVis-Tomato* and *en-Gal4 UAS-GFP/UAS-ChtVis-Tomato-control* prepupae (Fig. 2C,D). As expected, both green and red stripes were seen with the ChtVis-Tomato reporter. However, no red stripes were seen with ChtVis-Tomato-Control, which lacks the CBD (Fig. 1). This result confirmed that chitin binding was essential for the stripe fluorescence.

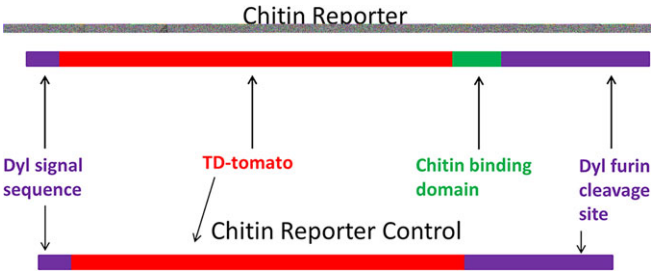


**Fig. 1. The segments of ChtVis-Tomato and ChtVis-Tomato-control reporter constructs.** The upper diagram shows the four segments of the ChtVis-Tomato reporter. Starting at the amino terminus (left) is the signal sequence from Dyl to provide for apical secretion, the td-Tomato fluorescent protein, the chitin-binding domain and the C terminal region of Dyl that contains a furin cleavage site. Below is shown the reporter control which differs by the omission of the chitin-binding domain.

As an alternative method to test whether the secreted reporter would undergo limited diffusion during cuticle formation within a cell, we generated flip-out clones expressing Gal4 that contained both *UAS-GFP* and *UAS-ChtVis-Tomato*. Clones were induced during the second day of pupal life and then not examined for another 24 h. This allowed us to label cells and/or sense organs (Fig. 3A-C) and also established that the reporter could be used to visualize chitin at later stages in pupal development (day 3) than is possible using an applied stain. It was apparent that the chitin reporter was located externally to the F-actin in the growing bristle (Fig. 3A-C) as would be expected if the reporter were binding to extracellular chitin. The reporter was also located externally to F-actin in developing wing hairs (Fig. 3D).

### The chitin reporter specifically stains chitin

In previous experiments where we stained fixed pupal wings (and other pupal epidermal tissues) with a tagged chitin binding protein, hair staining was first detected around 42 h after white prepupae (awp) and by about 44 h the staining was strong (Adler et al., 2013). To test the reporter we induced expression by putting the animals at 29.5°C for 4 h and then observed the pupae by *in vivo* imaging or by examining stained fixed tissues. Similar results were obtained in both sets of experiments. We observed strong hair fluorescence on the wing and thorax, and strong bristle fluorescence in slightly older animals (Fig. 2E; Fig. 3E,F, arrows). In previous experiments on fixed and stained material we described how chitin is prominently found in bands that run along the proximal distal axis of bristles (Nagaraj and Adler, 2012). This was also seen by *in vivo* imaging (Fig. 3E, arrow). By contrast, we did not observe any fluorescence in hairs or bristles in animals that were too young to have begun cuticle deposition, confirming the specificity of the reporter (this is described in more detail later). We also observed strong fluorescence of the pupal cuticle that surrounds the pupae (asterisks in Fig. S2A, Fig. S3H, Fig. S4). This was seen regardless of the time when the reporter was expressed. This observation was expected as the secreted reporter would be able to bind to the pupal cuticle being deposited from about 12-18 h awp. Using the reporter in both *in vivo* imaging and in fixed material we often observed a swirling banding pattern in the pupal cuticle sac that surrounds the wing (Fig. S2A). Similar swirling of fibers in abdominal pupal cuticle was also seen in TEM images (Fig. S2B). These observations seem likely to be a result of the pupal cuticle chitin fiber bundles being less densely packed than in the stronger and thicker adult cuticle, allowing us to see individual fiber bundles.

As a control to confirm that the chitin reporter was indeed binding to chitin, we also utilized the construct that lacked a CBD (Fig. 1). We detected no evident fluorescence of this control protein on hairs or bristles. Indeed, the bristles appeared as dark areas (Fig. 3G, arrow) surrounded by faint fluorescence throughout the liquid between the pupal cuticle and partially deposited adult cuticle. This was true at a variety of developmental stages (Fig. S3J,K, arrows).

As a further and more definitive test that the reporter was detecting chitin, we generated clones of cells that were homozygous

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

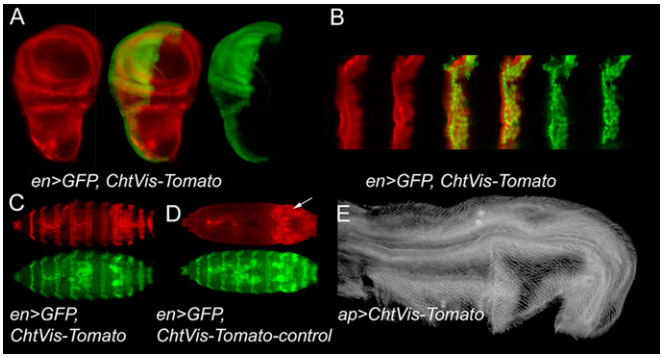


**Fig. 2. ChtVis-Tomato secreted apically.** (A) An *en-Gal4 UAS-GFP*/*UAS-ChtVis-Tomato* wing disc. Cytoplasmic GFP (green) accumulates only in the posterior compartment cells that express it as a result of the *en-Gal4* driver. The ChtVis-Tomato protein, which includes td-Tomato (red), accumulates throughout the disc in the space between the epithelial cells and the peripodial membrane as a result of being secreted apically. (B) Larval body wall from an *en-Gal4 UAS-GFP*/*UAS-ChtVis-Tomato* larva. Fluorescent stripes of both GFP and td-Tomato (from ChtVis-Tomato) are seen (two stripes in each image). The difference between this result and the one shown in A is that when ChtVis-Tomato is secreted apically it immediately binds to chitin in the larval cuticle to which the larval epidermal cells are connected. (C) An *en-Gal4 UAS-GFP*/*UAS-ChtVis-Tomato* prepupa shows stripes of both GFP and td-Tomato. Again, the secreted ChtVis-Tomato likely binds to chitin in the larval cuticle that overlays the epidermal cells. (D) An *en-Gal4 UAS-GFP*/*UAS-ChtVis-Tomato-control* prepupa shows stripes of GFP but not td-Tomato as the ChtVis-Tomato-Control lacks a CBD. The arrow points to the wing disc inside of the prepupa which contains the secreted reporter as in Fig. S3B. (E) A 50 h *ap-Gal4* /*UAS-ChtVis-Tomato* pupal wing. Hairs are marked by the chitin reporter.

for a mutation in *kkv*, the chitin synthase that synthesizes chitin found in cuticle. Because of the weakness of cuticle lacking in chitin, only animals with small *kkv* clones are viable. In adult wings, *kkv* hairs are almost invisible under a light microscope because of a lack of pigment, and the mutant hairs being flaccid and lying directly on the wing blade (Fig. 4C) (Adler et al., 2013). We previously published an experiment where we stained pupal wings with a fluorescently tagged chitin binding protein and observed a loss of staining that was restricted to the mutant cells (Adler et al., 2013). Such experiments are complicated by flattening of the wing cells beginning prior to the start of chitin deposition, and problems with immunostaining as a result of cuticle blocking access of the antibody to the clone marker. To lessen the accessibility problem, we used minimal fixation in experiments to test the specificity of the *in vivo* chitin reporter. This resulted in less than optimal, but still interpretable, morphology (e.g. hairs sometimes appeared to fall apart during the protocol, resulting in what appeared to be 'doubled' hairs). When we expressed the chitin reporter, but did not use immunostaining to mark the clone boundaries, we observed 'missing' hairs that were not detected by the reporter (Fig. 4A, asterisks). When we also used immunostaining to mark clone boundaries, we found that this lack of detection of the reporter in the hair was only seen in mutant cells (Fig. 4B,D, asterisks). This established that the chitin reporter specifically detects chitin.

As a test of whether ChtVis-Tomato could bind directly to chitin, we generated extracts of *ap>ChtVis-Tomato*, *ap>ChtVis-Tomato* and *Ore-R* wing discs and incubated the extracts with chitin magnetic beads. We then assayed the proteins bound to the beads by western blotting using an anti-Tomato monoclonal antibody. We found that ChtVis-Tomato bound, but ChtVis-Tomato-control did not (Fig. S5). Thus, we concluded from this test that the CBD present in ChtVis-Tomato is functional and leads to the reporter binding chitin.

A number of studies have used cuticle autofluorescence to analyze cuticle structure (Klaus et al., 2003; Zill et al., 2000; Haug et al., 2011). To determine if autofluorescence was impacting our studies we compared the fluorescence of a variety of ChtVis-Tomato-expressing cells and tissues to wild type. In all cases we found that autofluorescence was negligible compared with the fluorescence of ChtVis-Tomato (Fig. S3).

The previous experiments established that the chitin reporter was able to specifically detect chitin in developing *Drosophila* adult cuticle. The staining observed could represent the reporter stably binding to chitin or it could represent an equilibrium condition with reporter dynamically binding and releasing. To distinguish between these two possibilities we used the FRAP (fluorescence recovery after photobleaching) technique. If the reporter was stably bound, we predicted that after bleaching, recovery would be minimal as inactive reporter would block access of newly synthesized reporter to the chitin. By contrast, if the reporter was dynamically binding and releasing we predicted that recovery would be rapid and

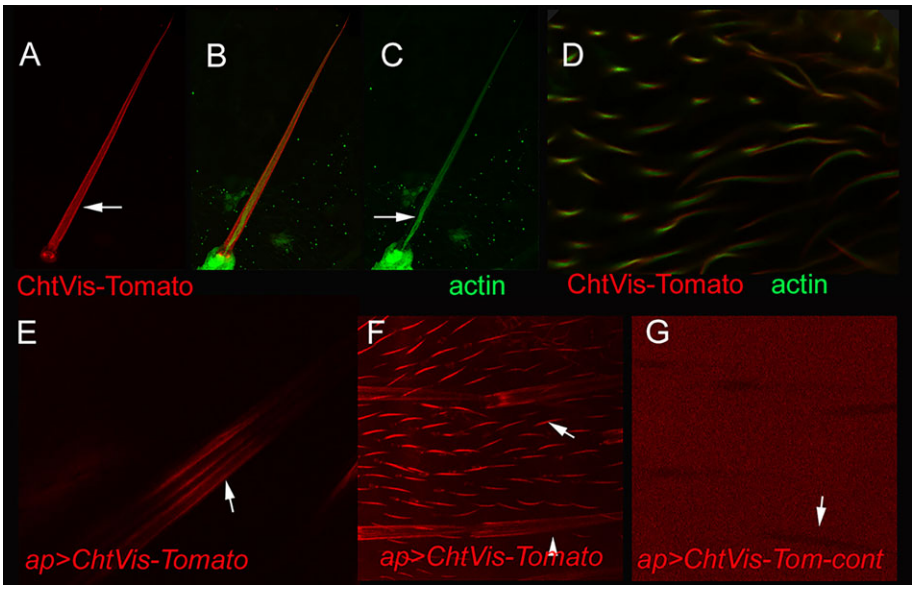


**Fig. 3. The chitin reporter labels hairs and bristles.** (A-C) Flip-out clone in an *Ay-Gal4 UAS-GFP*/*UAS-ChtVis-Tomato* pupae showing td-Tomato (A), and also stained with Alexa Fluor 488 phalloidin to show F-actin (C). Merge in (B), the arrow points to the bristle. Note that as expected, the actin is internal to the chitin reporter. (D) Small region of a 46 h *ap-Gal4*/*UAS-ChtVis-Tomato* pupal wing stained for td-Tomato and F-actin. Again, note in the hair that the actin is internal to the chitin reporter. (E) Small region of a bristle in a living 48 h *ap-Gal4*/*UAS-ChtVis-Tomato* pupa. The bands of chitin staining resemble those seen with a staining protocol (arrow). The bands represent folds in the bristle where the cuticle is thicker. (F) A region of the dorsal thorax of a living 43 h *ap-Gal4*/*UAS-ChtVis-Tomato* pupa. The arrow points to a hair and the arrowhead to a bristle. (G) A living 43 h *ap-Gal4*/*UAS-ChtVis-Tomato-control* pupa. Note the lack of staining of hairs or bristles (arrow). Indeed, the bristles appear as regions that lack the background fluorescence of the secreted UAS-ChtVis-Tomato-control.

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

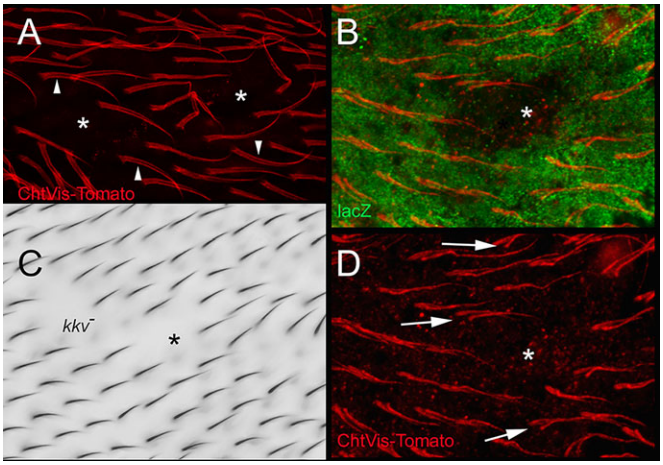


**Fig. 4. The ChtVis-Tomato is specific for chitin.** (A) A 45 h *ap-Gal4/UAS-ChtVis-Tomato; FRT82 actP-lacz/FRT82 kkv*$^{1}$ *e* pupal wing that contains two putative small *kkv*$^{1}$ clones (asterisks). The clone was not marked by *lacZ* expression and its presence is inferred from the locations where no hairs are labeled by ChtVis-Tomato. The wild-type hairs are stained by the ChtVis-Tomato (arrowheads). (B) A 45 h *ap-Gal4/UAS-ChtVis-Tomato; FRT82 actP-lacz/FRT82 kkv*$^{1}$ *e* pupal wing that contains a small *kkv*$^{1}$ clone marked by the loss of *lacZ* (green) (asterisk). The wild-type hairs are marked by the ChtVis-Tomato (red). (C) An adult wing with a several small *kkv*$^{1}$ clones (asterisk). The mutant hairs are not visible in this micrograph. Note similarity to panel A. (D) The same wing shown in B, but only showing the ChtVis-Tomato (red) channel. The clone is once again marked by an asterisk and hairs showing the double hair phenotype (see text) observed with this staining protocol are noted by an arrow.

complete as the bleached reporter was replaced by unbleached molecules. When we bleached the reporter on thoracic bristles, fluorescence substantially recovered in the bleached area within 18 s (Fig. 5, Movie 1). However, there was never a complete recovery of fluorescence. This implies that there are two pools of reporter in the bristle; one stably bound and the second continually binding and releasing from chitin (Fig. 5). Similar results were obtained in other FRAP experiments on developing bristles but we did not quantitatively analyze how the results might vary as a function of developmental stage or the position and size of the bleached region along the proximal distal axis.

### The chitin reporter can be used for long term *in vivo* imaging experiments

To determine if the chitin reporter was suitable for long term time lapse observations we examined the developing notum where the chitin reporter was expressed (Fig. 6). In previous experiments where we stained fixed tissue with a tagged chitin binding protein, we first observed chitin staining of bristles around 42 h awp (Nagaraj and Adler, 2012). For a brief time the staining was much brighter proximally but shortly afterwards we observed it in all positions along the bristle shaft. We also noted that hair staining began earlier than 42 h awp. In preliminary experiments we failed to see evidence of bristle fluorescence in pupal dorsal notum at 33 h. In such pupae the bristles could sometimes be detected in confocal optical sections by a lack of fluorescence in a field of faint fluorescence. These resembled images obtained when the control reporter lacking the CBD was expressed. We were able to observe hair fluorescence and there was a hint of bristle fluorescence in the notum of 39.5 h pupae (Fig. 6A,A′). The brightness of the reporter increased rapidly and by 42 h awp the hairs and by 44 h awp the bristles could be clearly observed (Fig. 6B,C,B′,C′). As

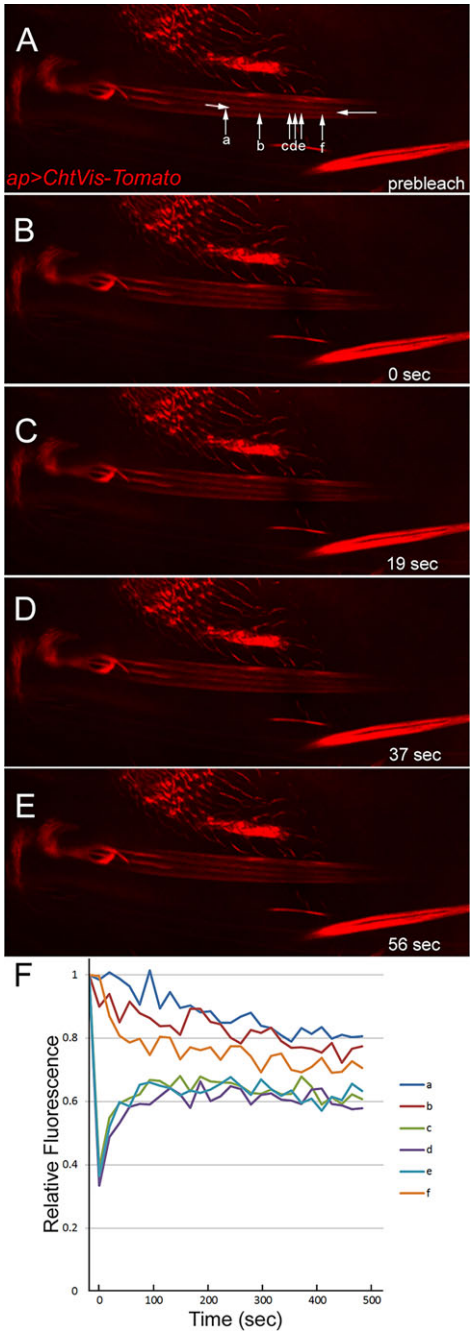


**Fig. 5. FRAP analysis of the chitin reporter in bristles.** (A-E) A 45 h *ap>ChtVis-Tomato* notum with a bristle in the center of the field. The bristle was bleached by several full power scans and then the recovery was followed over time. The horizontal arrow points to the band of ChtVis-Tomato fluorescence used for quantitation. The fluorescence was measured over time at six locations (a-f) along the bristle. Two were proximal to the bleached region (a,b), three were in the bleached region (c-e) and one was distal to the bleached region (f). (F) Quantification of fluorescence at the six positions in A-E. Note that in the bleached locations fluorescence never recovered to the level seen in the unbleached regions. The recovery that did take place was complete by 74 s and about half of it was seen at 18 s. The gradual decline in fluorescence over time in all samples after 64 s is a result of bleaching of td-Tomato by the confocal laser during image acquisition.

development proceeded fluorescence intensity increased until reaching a maximum around 63 h (Fig. 6D,D′). In 63 h pupae we also observed reporter fluorescence in the notum cuticle apart from the hairs and bristles. By 80 h awp the brightness of the reporter

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

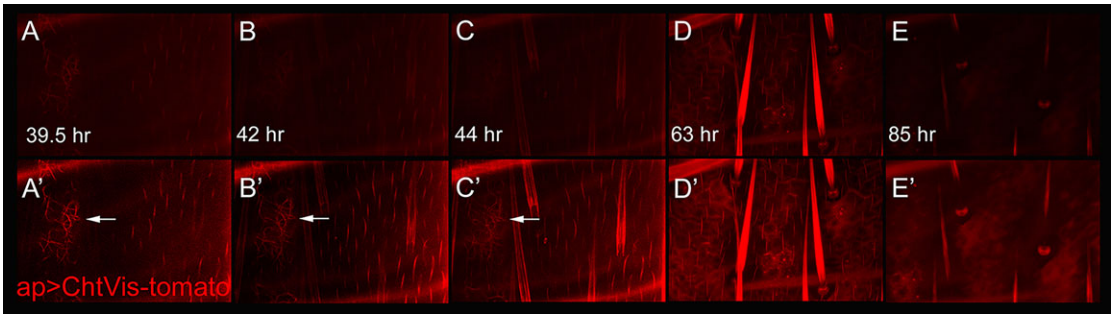


**Fig. 6. Performance of ChtVis-Tomato in time lapse experiments.** Part of the notum of an *ap-Gal4*/*UAS-ChtVis-Tomato* pupa followed from 39.5 to 85 h awp. The upper panels show images that were not enhanced to allow comparison of the relative brightness at different time points. These images were all acquired with the same microscope settings and are comparable. The lower panels show images where the brightness and contrast were increased. The arrows point to the putative chitin fibers discussed in the text. Note how the brightness is highest in the 63 h time point.

decreased (Fig. 6E,E′). At this time the adult cuticle begins to accumulate pigment and we suspect the absorbance of both excitation and emission light plays a key role in decreasing fluorescence. The loss of td-Tomato activity because of age or the developing cuticle environment might also be contributing to the decreased fluorescence. Our observations established that the chitin reporter is suitable for long term *in vivo* imaging of *Drosophila* pupae.

### Unexpected chitin fibers that are not integrated into cuticle

In previous experiments where we stained fixed pupal wings with a fluorescently tagged chitin binding protein we frequently observed stained fibrous material loosely attached to pupal wings. Their location was not random and they were primarily found in a number of stereotypic locations (see Fig. 7 for examples). This staining of fibrous material could also be seen when the chitin reporter was expressed (Fig. 7B,C). These fibrous deposits were not seen in 25 h pupal wings (Fig. 7A) so they are unlikely to be produced during the deposition of the pupal cuticle. By 29 h they could be seen in pupal wings and remained visible in 44 h pupal wings. Thus, the fibrous deposits could be detected ∼13 h prior to the time we first observed chitin staining of the hairs at 42 h. They could be detected for several hours after the start of cuticle deposition in the hairs but were lost by 54 h awp. We also observed similar fibers near the notum during *in vivo* imaging experiments in 34 h pupae (Fig. 6A′,B′,C′,D′), and as we followed such pupae we noticed the fluorescence decreased slightly from 39.5 to 44 h and was lost by 63 h awp. In *z*-sections it was clear that the fibers were apical to the apical surface of the wing cells but below the pupal cuticle (Fig. S4, arrows). Consistent with the fibrous material being chitin, it was not detected by our control reporter that lacked a CBD.

The fibrous material did not appear to be highly dynamic, at least during the time period when it was brightest. For example, in the *in vivo* imaging experiment shown in Fig. 6 the fibrous material appeared to be rather stable between 39.5 and 42 h. These observations suggested that chitin fibers of unknown function are formed in the space between the pupal cuticle and the wing cells prior to the start of chitin deposition in adult cuticle.

As an additional test to determine if the fibrous material was in fact chitin we tested if it was sensitive to chitinase (see Materials and Methods). In these experiments we expressed ChtVis-Tomato in pupal wings using *ap-Gal4*. Pupae were fixed prior to the start of hair chitin deposition and the wings dissected. Some of the wings were then treated with chitinase a, whereas controls were simply incubated in enzyme buffer. We imaged the wings at various times after the start of the experiment. The control wings did not show a substantial loss of fluorescence (Fig. 7E,E′,F), with the maximum fluorescence of the reporter decreasing ∼20% over more than 2 h of incubation. By contrast, in the wings treated with chitinase there was a dramatic loss of fluorescence (Fig. 7D,D′,F). This result is as expected if the chitin reporter signal results from it binding to chitin in the fibrous material. We cannot rule out the alternative explanation that proteases contaminating the enzyme preparation reduced the staining by digesting the reporter and not the chitin. However, we think this explanation is less likely as we did not see disintegration of the wing as we have in other experiments where we treated pupal wings with proteases for long periods of time.

### The chitin reporter labels tracheal chitin

The chitin reporter was designed to be used to study exoskeleton chitin. It was for this reason that we used the *dyl* gene, which functions in the deposition of the cuticular exoskeleton, as a source of the amino and carboxy terminal segments. In addition to the epidermis, the trachea is a second well-studied tissue that forms a chitin-containing cuticle (Devine et al., 2005). We first tested if staining in the trachea by the reporter was specific by comparing white prepupae that expressed either ChtVis-Tomato or the control construct lacking the CBD. Staining of the trachea was only seen when the CBD was present (Fig. S6A,B). Hence, it is also specific for tracheal chitin. We examined the reporter in trachea in more detail in an experiment where we used the *btl-Gal4* driver to simultaneously express the reporter and a cytoplasmic GFP. We dissected out trachea from *btl-Gal4 UAS-GFP/UAS-ChtVis-Tomato* 3rd instar larvae and examined these by confocal microscopy. ChtVis-Tomato labeled the apical edge of the tracheal cells as expected if it were binding to tracheal cuticle chitin (Fig. S6C,E). The taenidial folding pattern of the trachea (Matusek et al., 2006) was clearly revealed by reporter fluorescence (Fig. S6C,E, arrow). Observations of wild-type trachea that were not expressing ChtVis-tomato (Fig. S6D,F) established that tracheal cuticle autofluorescence was not significant in this experiment.

## DISCUSSION

### Uses of the reporter

The chitin reporter we described here should be useful for a wide variety of experiments involving cells and tissues that synthesize chitin. In addition to the cuticle synthesized by the adult epidermis, limited experiments indicate that the reporter will also work for examining the synthesis of larval and pupal cuticle and the morphogenesis of the tracheal cuticle. It could be very useful in screening experiments to discover unknown genes that have a function in chitin deposition or in the assembly of cuticle. With the

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

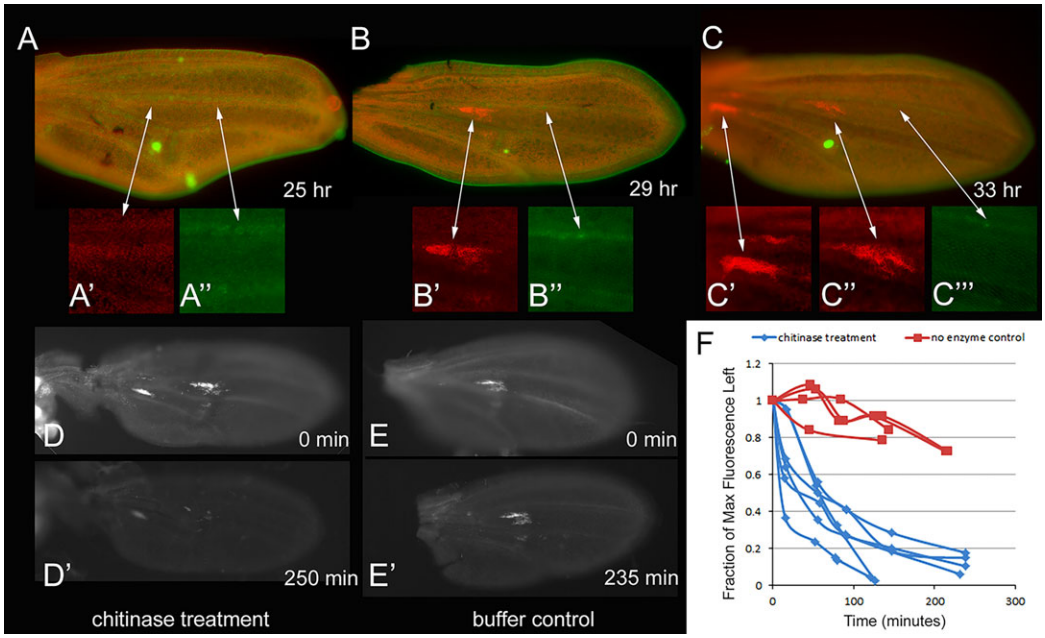


**Fig. 7. Putative chitin fibers are found prior to the start of hair formation but not in 25 h pupal wings.** (A-C) *ap-Gal4/UAS-ChtVis-Tomato* pupal wings lightly fixed and stained with Alexa Fluor 488 phalloidin. (A-A″) A 25 h pupal wing (A) lacks the chitin fibers (A′) and there are no hairs detected by actin staining (A″). (B-B″) A 29 h pupal wing (B) has one patch of chitin fibers (B′) and although there are no hairs seen by actin staining, the strong cell periphery staining typical of this stage can been seen (B″). (C-C‴) A 33 h pupal wing (C) has several patches of chitin fibers (C′,C″) and small hairs are seen with F-actin staining (C‴). (D,E) Mildly fixed *ap-Gal4/UAS-ChtVis-Tomato* pupal wings (32-36 h old) with patches of chitin fibers. (D) A wing just prior to the addition of chitinase. (D′) The same wing after incubation in chitinase for 250 min. (E) A control wing at the start of the experiment. (E′) The same wing after 235 min in chitinase buffer without any enzyme. (F) Quantification of the relative decrease in maximum fluorescence from the reporter over time for a number of wings with or without chitinase treatment.

chitin reporter, potential mutant animals could simply be examined live under a fluorescence microscope – a much easier and quicker screen than one that relies on dissecting and staining tissues prior to microscopic screening. Alternatively, the reporter could be used as a rapid way to characterize mutants identified using an alternative phenotype.

### How could ChtVis-Tomato be improved?

The reporter described in this paper was not our first attempt to develop a chitin reporter. Earlier reporters constructed in our lab differed in two ways. Our original reporter used m-Cherry as the fluorescent protein and we used the amino terminal segment of the cuticle protein encoding gene *dsx-c73A* (Andrew and Baker, 2008) to provide a signal sequence. This first generation reporter appeared to work but we did not extensively characterize it. The second generation reporter was much brighter and more photostable, making it much more convenient to use – particularly for time lapse *in vivo* imaging. We suspect that this was primarily as a result of the substitution of td-Tomato for m-Cherry but we did not test reporters that contained only one change. There are other possible future changes that might also result in improvements to the reporter. Our FRAP data suggested the protein binds and releases from chitin with a short half-life. This was done at an early time in bristle chitin deposition and it is possible that at a later stage the tight packing of the cuticle would result in the reporter not being able to diffuse away from the chitin it was bound to even if it was released. We suspect that a reporter that contained multiple CBDs would bind more tightly and be less likely to be released and bind again. A stably bound reporter would be preferable for some types of experiments where temporal aspects of chitin deposition were important. Another way it might be valuable to modify the reporter would be to include sequences that facilitate detection under an electron microscope (Gaietta et al., 2002; Lam et al., 2015; Shu et al., 2011).

Our data cannot rule out the possibility that ChtVis-Tomato could bind to other carbohydrates, although the lack of staining of cuticle synthesized by *kkv* mutant wing cells argues that such carbohydrates are not present in fly wing cuticle. Our success in the development and use of ChtVis-Tomato for chitin detection *in vivo* suggests that it would be useful to pursue similar strategies for detecting other carbohydrate polymers, such as cellulose.

### Pupal cuticle and chitin deposition

In a number of experiments we noted that the pupal cuticle was labeled by ChtVis-Tomato and that we were able to see spirals of reporter that presumably represented spirals of chitin bundles. Consistent with this interpretation, chitin spirals were prominent in images of pupal cuticle obtained by TEM. In our experience with *Drosophila*, adult cuticle layers of chitin are found but the density of the cuticle makes the morphology less clear cut. Based on this we suggest that pupal epidermis and cuticle would be a favorable model system for studying chitin deposition in insect cuticle.

### Use in other organisms

We developed the chitin reporter for use in *Drosophila* but expect that it would be useful in a wider range of organisms. We used the *dyl* backbone to provide two key components of the system. One was a signal sequence that would guide apical secretion, as chitin and cuticle are secreted apically. The second was the putative furin cleavage site. If the reporter was tethered to the plasma membrane it might interfere with the proper deposition of cuticle. As *dyl* is conserved in a wide variety of insects, we expect that ChtVis-Tomato would work in other insects. There are *dyl* homologs in non-insect arthropods such as *Daphnia*, making it probable that the reporter would also work in arthropods. Although there are *dyl* homologs in nematodes, they are thought to function in the formation of the hypoderm cuticle which does not contain chitin

Development (2015) 142, 3974-3981 doi:10.1242/dev.126987

(Sapio et al., 2005). There is chitin in the egg shell and pharynx (Zhang et al., 2005) but it is not known if one of the *dyl* homologs is expressed in the tissues that deposit this chitin or if our reporter would be secreted apically in these tissues. Thus, it is possible that a suitable chitin reporter for nematodes might need to be modified from the one we have described.

### Novel fibrous material

The detection of fibrous material stained by both the chitin reporter and by a fluorescently tagged chitin binding protein was not expected and to our knowledge has not been described previously. It was lost after treatment with chitinase and thus seems likely to be composed of chitin. It was located between the pupal cuticle and the wing/thoracic cells and was detected in wing preparations ~13 h prior to the time when we first detected chitin in wing hairs. It was gradually lost as development proceeded. The function of this fibrous material, if any, can only be guessed at. It might serve to sequester chitinase found in the liquid between the pupal cuticle and the epidermal cells and hence help to protect chitin deposited early in adult cuticle deposition. It would not be surprising if chitinase was found there as it could be needed earlier to facilitate the release of the epidermal cells from the pupal cuticle (Turner and Adler, 1995). It is uncertain if this fibrous material is found in insects other than *Drosophila melanogaster*, although that seems likely. Given that chitin is considered insoluble, its presence in a few locations suggests either that it is synthesized by cells only in those locations or that there is a chitin binding protein that both renders it soluble and targets it to those locations. Further studies will be needed to answer these questions.

## MATERIALS AND METHODS

### Fly genetics

The UAS/Gal4 system was used to direct the expression of the chitin reporter (Brand and Perrimon, 1993). In most experiments reported in this paper we generated and examined *w, UAS-ChtVis-Tomato/ap-Gal4 pTub-Gal80$^{ts}$* (McGuire et al., 2004) flies. They were grown for most of their development at 25°C or 21°C, temperatures at which the expression of the reporter is low. White prepuae were collected and aged for the desired time and then shifted to 29.5°C for 4 h to inactivate the ts Gal80 protein, allowing high level expression of the reporter. The pupae were then prepared for imaging. Several other *Gal4* drivers were used in some experiments. These include AyGal4 where a heat shock-mediated recombination at FRT sites resulted in the expression of Gal4, *ptc-Gal4*, *en-Gal4* and *btl-Gal4*. We also used *ap-Gal4* without *pTub-Gal80$^{ts}$* in some experiments.

### Microscopy

*In vivo* imaging was done as described previously (Nagaraj and Adler, 2012). Aged pupae were placed onto double-sided sticky tape on a microscope slide. Silicone rubber spacers were placed onto the slide and the pupal cuticle was removed locally or entirely. A coverslip with a small amount of halocarbon oil was placed onto the supports. The halocarbon oil created a good optical connection between the pupae and the coverslip. In some experiments the animals were incubated for various lengths of time at 25°C before imaging. In some of the *in vivo* imaging experiments the animals were moved back to a moist chamber in a 25°C incubator between imaging sessions. Most of the *in vivo* imaging experiments carried out in this study were done on the notum (dorsal thorax) as this is both a convenient tissue to examine and it allows us to image the development of hairs (trichomes), bristles and simple thoracic cuticle at one time.

The majority of imaging, and all of the *in vivo* imaging, was performed on a Zeiss 780 confocal microscope in the Keck Center for Cellular Imaging. A few preliminary experiments utilized a Zeiss 510 confocal microscope.

Adult wings were mounted in Euparal and examined on a Zeiss Axioskop 2 microscope and images obtained using a Diagnostic Instruments Spot-RT camera. This microscope and camera were also used to obtain low magnification images of stained pupal wings.

TEM was done at the University of Virginia Advanced Microscopy Center using standard techniques.

### Staining protocol

Pupal wings (or thoraces) were fixed with 3.7% paraformaldehyde, dissected in PBS, and then stained using standard procedures (Adler et al., 2004).

### Test of chitinase sensitivity of fibrous material detected by the reporter

Pupae (28-34 h awp) were fixed in 3.7% paraformaldehyde and pupal wings dissected in PBS, then placed into a shallow depression slide in chitinase buffer (50 mM potassium phosphate, pH 6.0). The pupal wings were imaged at 10× magnification by epifluorescence on a Zeiss Axioskop 2 microscope equipped with a Diagnostic Instruments Spot RT digital camera. The same exposure time was used for all wings in an experiment. After the original imaging, the slides were moved to a moist chamber and chitinase (Sigma-Aldrich, C6137, 0.5 units/µl) in buffer was added to the experimental group, but not the control group. At various times after the start of the experiment additional images were taken to follow the loss of the chitin staining fluorescence. The images were analyzed using ImageJ (National Institutes of Health). The pupal wings were floating in buffer in the depression slide and their movement in and out of the moist chamber caused changes in position so that not all images of any particular wing were taken at identical orientations. We analyzed changes over time both for maximum fluorescence intensity and integrated intensity in the fibrous area. Although similar results were obtained for both, we have reported the maximum intensity values as we felt this would be less sensitive to the orientation issues.

### Biochemical test of ChtVis-Tomato binding to chitin

Wing discs were dissected from *ap>ChtVis-Tomato*, *ap>ChtVis-Tomato-control* and *Ore-R* third instar larvae. One hundred discs were homogenized in 1.1 ml CBD column binding buffer (500 nM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.05% Trition X-100, pH 8.0 at 25°C) and centrifuged to remove debris. Washed chitin magnetic beads (New England Biolabs E8036S), or His-6-binding magnetic beads as a control (Anti-His tag magnetic bead: MBL D291-9), in 50 µl aliquots were incubated with 500 µl of disc extract for 4 h at 4°C. The beads were collected in a magnetic binding rack and washed several times. Proteins were eluted with 100 µl of SDS sample buffer at 100°C for 10 min. The samples were then analyzed by western blotting using an anti-tdTomato monoclonal antibody (1:2000; ORIGENE, TA180009) and goat anti-mouse IRDye 800CW (1:10,000; LI-COR, 926-32210) secondary antibody. The blots were visualized with a LI-COR Odyssey.

### Construction of ChtVis-Tomato

pWALIUM10-moe was used as a vector for the reporter. The signal sequence from *dsc73* was PCR amplified using oligonucleotides that incorporated *Eco*RI and *Nde*I restriction sites. The mCherry sequence was amplified to incorporate *Nde*I and *Bgl*II restriction sites. The CBD of the *Bacillus circulans* chitinase A1 gene (New England Biolabs) was amplified to incorporate *Bgl*II and *Xba*I restriction sites. The sequences were double-digested overnight using the respective enzymes, purified and ligated at 4°C at a 1:1:1 molar ratio. Subsequent PCR was performed with 1 µl of the ligation mixture and primers specific to the sequence ends (*Eco*RI and *Xba*I). This specific band was then purified from agarose gel and double digested. The insert was ligated to double-digested pWALIUM10-moe. This initial reporter plasmid was modified to make ChtVis-Tomato (Fig. S1). The tdTomato sequence (from FUtdTW, Addgene) was amplified with the same restriction sites as previously used for mCherry, and it was then used to replace the mCherry sequence using standard cloning techniques. A signal sequence from *dyl* was amplified from cDNA with the addition of *Nde*I and *Bgl*II restriction sites. TdTomato-CBD was amplified with *Bgl*II and *Xho*I restriction sites. The *dyl* tail sequence was amplified, truncating at the putative furin cleavage site with *Xho*I and *Xba*I restriction sites. For the control construct, tdTomato was cloned to contain *Nde*I and *Bgl*II restriction sites, and the *dyl* tail to contain *Bgl*II and *Xba*I sites. The sequences were assembled and subcloned using PCR as described above. Where necessary short linkers

were added to keep the gene in frame. The constructs were integrated into the fly genome at the VK00001 site (Venken et al., 2006). The sequences of the final plasmids are provided in Fig. S7 and the sequences of the primers used in the construction are found in Table S1. pWalium-ChtVis-Tomato is available from Addgene (72102).

#### Acknowledgements
We thank FlyBase and the Bloomington Drosophila Stock Center, which were essential for our research. We thank the S. Segrist lab (University of Virginia, VA, USA) for the btl-Gal4 stock. We also want to thank the Keck Center for Cellular Imaging where the confocal imaging experiments were carried out and the University of Virginia Advanced Microscopy facility where the electron microscopy was done.

#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
P.N.A. conceived and directed the project. L.F.S. devised and constructed the reporter. All of the authors carried out and analyzed experiments to characterize the effectiveness of ChtVis-tdTomato. P.N.A. and L.F.S. both did extensive work in writing the manuscript and in preparing figures.

#### Funding
This work was supported by a grant from the National Institutes of Health [GM037163 to P.N.A.]; by funds from the William R. Kenan Jr. Professorship; and by funds from the Office of the Associate Provost for Research for work on University Advisory Committees. Deposited in PMC for release after 12 months.

#### Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126987/-/DC1

#### References
**Adler, P. N., Zhu, C. and Stone, D.** (2004). Inturned localizes to the proximal side of wing cells under the instruction of upstream planar polarity proteins. *Curr. Biol.* **14**, 2046-2051.
**Adler, P. N., Sobala, L. F., Thom, D. and Nagaraj, R.** (2013). dusky-like is required to maintain the integrity and planar cell polarity of hairs during the development of the *Drosophila* wing. *Dev. Biol.* **379**, 76-91.
**Andrew, D. J. and Baker, B. S.** (2008). Expression of the *Drosophila* secreted cuticle protein 73 (dsc73) requires Shavenbaby. *Dev. Dyn.* **237**, 1198-1206.
**Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., Lorenzen, M. D., Kanost, M. and Beeman, R. W.** (2005). The Tribolium chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. *Insect Mol. Biol.* **14**, 453-463.
**Brand, A. H. and Perrimon, N.** (1993). Targetted gene expression as a means of altering cell fate and generating dominant phenotypes. *Development* **118**, 401-415.
**Cornman, R. S.** (2010). The distribution of GYR- and YLP-like motifs in *Drosophila* suggests a general role in cuticle assembly and other protein-protein interactions. *PLoS ONE* **5**, e12536.
**Devine, W. P., Lubarsky, B., Shaw, K., Luschnig, S., Messina, L. and Krasnow, M. A.** (2005). Requirement for chitin biosynthesis in epithelial tube morphogenesis. *Proc. Natl. Acad. Sci. USA* **102**, 17014-17019.
**dos Santos, G., Schroeder, A. J., Goodman, J. L., Strelets, V. B., Crosby, M. A., Thurmond, J., Emmert, D. B., Gelbart, W. M.** and **the FlyBase Consortium** (2015). FlyBase: introduction of the *Drosophila* melanogaster Release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Res.* **10**, D690-D697.
**Fernandes, I., Chanut-Delalande, H., Ferrer, P., Latapie, Y., Waltzer, L., Affolter, M., Payre, F. and Plaza, S.** (2010). Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. *Dev. Cell* **18**, 64-76.
**Gagou, M. E., Kapsetaki, M., Turberg, A. and Kafetzopoulos, D.** (2002). Stage-specific expression of the chitin synthase DmeChSA and DmeChSB genes during the onset of *Drosophila* metamorphosis. *Insect Biochem. Mol. Biol.* **32**, 141-146.
**Gaietta, G., Deerinck, T. J., Adams, S. R., Bouwer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y. and Ellisman, M. H.** (2002). Multicolor and electron microscopic imaging of connexin trafficking. *Science* **296**, 503-507.
**Gangishetti, U., Breitenbach, S., Zander, M., Saheb, S. K., Müller, U., Schwarz, H. and Moussian, B.** (2009). Effects of benzoylphenylurea on chitin synthesis and orientation in the cuticle of the *Drosophila* larva. *Eur. J. Cell Biol.* **88**, 167-180.
**Haug, J. T., Haug, C., Kutschera, V., Mayer, G., Maas, A., Liebau, S., Castellani, C., Wolfram, U., Clarkson, E. N. K. and Waloszek, D.** (2011). Autofluorescence imaging, an excellent tool for comparative morphology. *J. Microsc.* **244**, 259-272.
**Jovine, L., Qi, H., Williams, Z., Litscher, E. S. and Wassarman, P. M.** (2004). A duplicated motif controls assembly of zona pellucida domain proteins. *Proc. Natl. Acad. Sci. USA* **101**, 5922-5927.
**Klaus, A. V., Kulasekera, V. L. and Schawaroch, V.** (2003). Three-dimensional visualization of insect morphology using confocal laser scanning microscopy. *J. Microsc.* **212**, 107-121.
**Lam, S. S., Martell, J. D., Kamer, K. J., Deerinck, T. J., Ellisman, M. H., Mootha, V. K. and Ting, A. Y.** (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nat. Methods* **12**, 51-54.
**Matusek, T., Djiane, A., Jankovics, F., Brunner, D., Mlodzik, M. and Mihály, J.** (2006). The *Drosophila* formin DAAM regulates the tracheal cuticle pattern through organizing the actin cytoskeleton. *Development* **133**, 957-966.
**McGuire, S. E., Mao, Z. and Davis, R. L.** (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. *Sci. STKE* **2004**, p16.
**Merzendorfer, H.** (2013). Chitin synthesis inhibitors: old molecules and new developments. *Insect Sci.* **20**, 121-138.
**Merzendorfer, H. and Zimoch, L.** (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J. Exp. Biol.* **206**, 4393-4412.
**Michels, J. and Büntzow, M.** (2010). Assessment of Congo red as a fluorescence marker for the exoskeleton of small crustaceans and the cuticle of polychaetes. *J. Microsc.* **238**, 95-101.
**Molano, J., Bowers, B. and Cabib, E.** (1980). Distribution of chitin in the yeast cell wall. An ultrastructural and chemical study. *J. Cell Biol.* **85**, 199-212.
**Moussian, B.** (2013). The apical plasma membrane of chitin-synthesizing epithelia. *Insect Sci.* **20**, 139-146.
**Moussian, B., Schwarz, H., Bartoszewski, S. and Nüsslein-Volhard, C.** (2005). Involvement of chitin in exoskeleton morphogenesis in *Drosophila* melanogaster. *J. Morphol.* **264**, 117-130.
**Nagaraj, R. and Adler, P. N.** (2012). Dusky-like functions as a Rab11 effector for the deposition of cuticle during *Drosophila* bristle development. *Development* **139**, 906-916.
**Sapio, M. R., Hilliard, M. A., Cermola, M., Favre, R. and Bazzicalupo, P.** (2005). The Zona Pellucida domain containing proteins, CUT-1, CUT-3 and CUT-5, play essential roles in the development of the larval alae in Caenorhabditis elegans. *Dev. Biol.* **282**, 231-245.
**Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E. and Tsien, R. Y.** (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567-1572.
**Shu, X., Lev-Ram, V., Deerinck, T. J., Qi, Y., Ramko, E. B., Davidson, M. W., Jin, Y., Ellisman, M. H. and Tsien, R. Y.** (2011). A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. *PLoS Biol.* **9**, e1001041.
**Sloat, B. F. and Pringle, J. R.** (1978). A mutant of yeast defective in cellular morphogenesis. *Science* **200**, 1171-1173.
**Tang, W. J., Fernandez, J. G., Sohn, J. J. and Amemiya, C. T.** (2015). Chitin is endogenously produced in vertebrates. *Curr. Biol.* **25**, 897-900.
**Taylor, M. J., Ponikau, J. U., Sherris, D. A., Kern, E. B., Gaffey, T. A., Kephart, G. and Kita, H.** (2002). Detection of fungal organisms in eosinophilic mucin using a fluorescein-labeled chitin-specific binding protein. *Otolaryngol. Head Neck Surg.* **127**, 377-383.
**Teparić, R. and Mrša, V.** (2013). Proteins involved in building, maintaining and remodeling of yeast cell walls. *Curr. Genet.* **59**, 171-185.
**Tronchin, G., Poulain, D., Herbaut, J. and Biguet, J.** (1981). Localization of chitin in the cell wall of Candida albicans by means of wheat germ agglutinin. Fluorescence and ultrastructural studies. *Eur. J. Cell Biol.* **26**, 121-128.
**Turner, C. M. and Adler, P. N.** (1995). Morphogenesis of *Drosophila* pupal wings in vitro. *Mech. Dev.* **52**, 247-255.
**Venken, K. J. T., He, Y., Hoskins, R. A. and Bellen, H. J.** (2006). P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. *Science* **314**, 1747-1751.
**Vincent, J. F.** (2005). Deconstructing the design of a biological material. *J. Theor. Biol.* **236**, 73-78.
**Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. and Tanaka, H.** (1994). The roles of the C-terminal domain and type III domains of chitinase A1 from Bacillus circulans WL-12 in chitin degradation. *J. Bacteriol.* **176**, 4465-4472.
**Willis, J. H.** (2010). Structural cuticular proteins from arthropods: annotation, nomenclature, and sequence characteristics in the genomics era. *Insect Biochem. Mol. Biol.* **40**, 189-204.
**Zakrzewski, A.-C., Weigert, A., Helm, C., Adamski, M., Adamska, M., Bleidorn, C., Raible, F. and Hausen, H.** (2014). Early divergence, broad distribution, and high diversity of animal chitin synthases. *Genome Biol. Evol.* **6**, 316-325.
**Zhang, Y., Foster, J. M., Nelson, L. S., Ma, D. and Carlow, C. K. S.** (2005). The chitin synthase genes chs-1 and chs-2 are essential for C. elegans development and responsible for chitin deposition in the eggshell and pharynx, respectively. *Dev. Biol.* **285**, 330-339.
**Zill, S., Frazier, S. F., Neff, D., Quimby, L., Carney, M., DiCaprio, R., Thuma, J. and Norton, M.** (2000). Three-dimensional graphic reconstruction of the insect exoskeleton through confocal imaging of endogenous fluorescence. *Microsc. Res. Tech.* **48**, 367-384.