Mechano-chemical enforcement of tendon apical ECM into nano-filaments during *Drosophila* flight muscle development

**Graphical Abstract**

**Developing muscle-tendon system**

**Authors**
Wei-Chen Chu, Shigeo Hayashi

**Correspondence**
shigeo.hayashi@riken.jp

**In Brief**
Chu and Hayashi show that the *Drosophila* tendon apical ZPD protein Dpy anchors the tendon cells to the pupal cuticle during flight muscle development. Dpy remodels to a filamentous organization under tension. The tensile strength of Dpy filaments is enhanced by another apical ZPD protein, Qsm, to ensure proper muscle-tendon system development.

**Highlights**
- Tendon cells connect to the pupal cuticle through the Dpy apical ECM matrix
- Dpy matrix undergoes a filament-like transformation during muscle development
- Muscle tension applied to the tendon promotes remodeling of Dpy filaments
- Diffusible apical ECM protein Qsm enhances the tensile strength of Dpy filaments

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Mechano-chemical enforcement of tendon apical ECM into nano-filaments during Drosophila flight muscle development

Wei-Chen Chu1 and Shigeo Hayashi1,2,3,4
1Laboratory for Morphogenetic Signaling, RIKEN Center for Biosystems Dynamics Research (BDR), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan
2Department of Biology, Kobe University Graduate School of Science, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan
3Lead contact
*Correspondence: shigeo.hayashi@riken.jp
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SUMMARY

Contractile tension is critical for musculoskeletal system development and maintenance. In insects, the muscular force is transmitted to the exoskeleton through the tendon cells and tendon apical extracellular matrix (ECM). In Drosophila, we found tendon cells secrete Dumpy (Dpy), a zona pellucida domain (ZPD) protein, to form the force-resistant filaments in the exuvial space, anchoring the tendon cells to the pupal cuticle. We showed that Dpy undergoes filamentous conversion in response to the tension increment during indirect flight muscle development. We also found another ZPD protein Quasimodo (Qsm) protects the notum epidermis from collapsing under the muscle tension by enhancing the tensile strength of Dpy filaments. Qsm is co-transported with Dpy in the intracellular vesicles and diffuses into the exuvial space after secretion. Tissue-specific qsm expression rescued the qsm mutant phenotypes in distant tissues, suggesting Qsm can function in a long-range, non-cell-autonomous manner. In the cell culture assay, Qsm interacts with Dpy-ZPD and promotes secretion and polymerization of Dpy-ZPD. The roles of Qsm underlies the positive feedback mechanism of force-dependent organization of Dpy filaments, providing new insights into apical ECM remodeling through the unconventional interaction of ZPD proteins.

INTRODUCTION

Mechanical force of muscles is transmitted to the skeleton through tendons. Loss of applied force results in developmental atrophy of tendon tissues and severe motor disorder.1,2 The mechanical tension is critical to myotendinous system development and maintenance, in part via regulating the secretion and organization of the collagen extracellular matrix (ECM) of the vertebrate tendon.3,4 During muscle development, tendon cells and ECM develop resistance to increasing contractility in the developing muscles to keep the musculoskeletal system from collapsing. However, the process by which mechanical force regulates the dynamic organization of tendon ECM in vertebrates is unclear. Here, we used Drosophila indirect flight muscles (IFM) and the associated tendon cells as a model to identify key molecular components of tendon ECM maturation.

Drosophila IFM are encased in the mesothoracic body wall. Based on their position, IFM are classified into two groups: the dorsal-longitudinal muscles (DLM) and the dorsal-ventral muscles (DVM).5,6 The muscle-tendon and tendon-cuticle attachments must be formed firmly to ensure efficient transmission of muscular force. While the developmental process of the muscle-tendon attachment involving microtubules, integrins, and basement membrane components has been well described in Drosophila,7,9 only little is known about tendon-cuticle attachment. A fibrous ECM, so-called tonofilaments, is associated with the site of tendon cell anchorage to the cuticle. Tonofilaments have been described as an electron-dense filamentous structure when observed under a transmission electron microscope, and they were found in the cuticle of muscle insertion sites at the embryonic,10,11 larval,12,13 or adult14 stages in Drosophila.

It was shown that tonofilaments fail to anchor the cuticle in the mutants of zona pellucida domain (ZPD) proteins, including papiliote15,16, piopio17, and dumpy18,19. ZPD is a conserved protein oligomerization domain composed of around 260 amino acids.19–21 A Drosophila ZPD protein Dumpy (Dpy) is a giant protein of up to 22,949 amino acid long forming a fiber structure at epidermal-cuticle attachment sites.18 Dpy matrix was shown to contribute to the mechanical integrity of elastic apical ECM to support tracheal tube extension in embryos19,20 and to anchor the wing margin to cuticles to help in the elongation of wing blade during the pupal stage.21,22 Some dpy hypomorphic mutants exhibit ectopic epidermal invaginations at the muscle insertion sites23,24 where high tension is loaded during IFM development.23,25 These findings imply that Dpy plays a role to resist increasing tension during flight muscle maturation.

Here we showed that the Dpy matrix connecting the tendon cells to the pupal cuticle is converted into a tensile, filamentous structure under tension buildup from the developing IFM. The tensile strength of Dpy filaments is enhanced by another ZPD protein Quasimodo (Qsm), which helps the secretion of Dpy. This work provides a novel mechano-chemical mechanism of...
ECM remodeling during the formation of mechanically robust tendon apical ECM during muscle development.

RESULTS

Force-dependent formation of Dpy filaments

To study the localization of Dpy in the notum muscle insertion sites, pupae of the Dpy-YFP knockin strain\textsuperscript{26,27} were imaged live at the anterior notum margin where DLMs form tendon structures (Figure 1A–E). Tendon cells labeled with stripe (sr) enhancer are specialized parts of the body wall epidermis that simultaneously connect with developing muscles through basolateral elongations in the body cavity and with the pupal cuticle (apolysis process) to open an extracellular space between the pupal cuticle and tendon/epidermis, known as exuvial space (Figure 1A and 1C–1E; 24–34 h APF). It has been shown that the mechanical tension in the muscle-tendon system is gradually built up during the myofibril maturation processes.\textsuperscript{9,28}

**Figure 1. Dpy filaments formation in response to tension**

(A) Organization of muscle-tendon-cuticle attachment in the pupa (about 30 h APF). The box on the lateral view (left) marks the anterior attachment site of DLM, which is schematized on the right side. The dashed blue box indicates the imaged area for the rest of the panels. (B–E) Time course of Dpy filaments formation visualized by Airyscan live imaging of Dpy-YFP (green) and tendon-specific sr-Gal4-driven mCherry-CAAX (magenta). Upon increasing tension of developing DLM, filamentous Dpy accumulates in the exuvial space (e) that opens between the pupal cuticle (c) and tendon cells (magenta; see Figure S1 for quantification). White arrowhead in (E) indicates Dpy matrix attached to non-tendon epidermis. (F, F’, and F’’) An apical secreted chitin reporter ChtVIS-Tomato,\textsuperscript{29} driven by sr-Gal4, labels pupal cuticle (c) but not Dpy filaments in the exuvial space. (G and H) Dpy organization in the Drok knockdown pupae at 32 h and 35 h APF. (G) MTJ are partially retained, and Dpy-YFP appears filamentous. (H) MTJ are completely broken. Dpy-YFP shows an aggregated appearance. (I and J) Dpy-YFP filaments appear short in conditions of reduced mechanical tension. Muscle-tendon junction was broken by muscle-specific Mef2-Gal4 driven kon-IR (I). Flight muscle was ablated by adult muscle precursor-specific 1151-Gal4 driven N\textsuperscript{ext} (J). Scale bar, 10 \textmu m.
During this period of maturation, we noticed a prominent change in the pattern of pupal tendon Dpy matrix (Figure 1B–1E). In the pupal cuticle, Dpy-YFP was distributed as a mesh-like structure colocalizing with chitin (Figure 1B–1F). Additionally, we observed a new pattern of Dpy-YFP localization appearing in the exuvial space over tendon cells that developed from a mesh-like structure to elongated filaments unassociated with chitin (Figure 1C–1F). Notably, the Dpy filaments are oriented along the axis of the elongated tendon cells pulled by the developing muscle (Figure 1E). Using OrientationJ analysis, we found a positive correlation between tension increment, Dpy-YFP orientation, and signal length (coherency) as the exuvial space increased (Figure S1). Further, to test whether the exuvial Dpy-YFP pattern is influenced by tension, we performed tendon cell-specific knockdown of Drok, which caused the breakdown of muscle-tendon-junction (MTJ) under the muscle tension. Before the MTJ breakdown, the exuvial Dpy-YFP still formed filamentous structures (Figure 1G). However, after the MTJ breakdown, the exuvial space was narrow, and Dpy filaments accumulated densely (Figure 1H), suggesting that tension in the muscle-tendon system pulled the exuvial Dpy matrix. When IFM were disrupted before the high-tension stage by muscle-specific knockdown of kon-tiki (kon), which is required for MTJ maturation, or by overexpression of constitutively activated form of Notch (N\textsuperscript{Act}), which inhibits the differentiation of IFM, the exuvial Dpy matrix failed to elongate (Figures 1I and 1J). These results suggest that the Dpy matrix undergoes a remodeling process in response to the mechanical tension from the developing muscle-tendon system.

Synergetic functions of dpy and qsm

Hypomorphic dpy mutants show phenotypes of epidermal invaginations or elevations on the notum at the muscle insertion sites of DLM (comma) and DVM (vortex, Figure 2D, 2D’, S2A, and S2A’) (Figures 2E and 2I). Another ZPD protein qsm mutant also showed the comma and vortex phenotypes. Null qsm\textsuperscript{F1/F0} allele created by genome editing (Figure 2A) were viable and phenocopied the previously reported qsm and the dpy\textsuperscript{ov1} mutant phenotypes (Figures 2D–2D’, 2E–2’D, S2A–S2B, and S2A’–S2B’). Null qsm (32 h APF) precedes that of maximal Dpy matrix length in the qsm mutant, which was not observed in the control (Figures 2B and 2E). The length of the tendon cells also increased to reach the maximum at 32 h APF and gradually returned to the original length (Figures 2A and 3H). In the qsm mutant, Dpy matrix elongated further, reaching the maximum at 34 h APF and shortened afterward (Figures 3B and 3G). The timing of maximal tendon cell length (32 h APF) precedes that of maximal Dpy matrix length in the qsm mutant (34 h APF). This is the timing of the onset of epidermal indentation observed in dpy mutants with comma phenotype. This correlation suggests that the tension in the muscle-tendon system caused the excessive elongation of exuvial Dpy filaments in the qsm mutants. Airyscan imaging carried out 33-34 h APF showed overstretched exuvial Dpy filaments in the qsm mutant, which were not observed in the control (Figures 3C–3F). These results suggest that the resilience of exuvial Dpy filaments is reduced in the qsm mutant, causing the adhesion of the tendon epidermis to the pupal cuticle to fail under the tension from the developing flight muscles.

Qsm modulates the resilience of Dpy filaments

To assess the possible effect of Qsm on Dpy matrix properties, we performed time-lapse live imaging of the anterior tendon cells of DLM in Dpy-YFP pupae. During a 14 h recording, the length of Dpy matrix steadily increased from 10 μm to 30 μm in the control pupa (Figures 3A and 3G; Video S1). In the qsm mutant, Dpy matrix elongated further, reaching the maximum at 34 h APF and shortened afterward (Figures 3B and 3G; Video S1). The timing of maximal tendon cell length (32 h APF) preceded that of maximal Dpy matrix length in the qsm mutant (34 h APF). This is the timing of the onset of epidermal indentation observed in dpy mutants with comma phenotype. This correlation suggests that the tension in the muscle-tendon system caused the excessive elongation of exuvial Dpy filaments in the qsm mutants. Airyscan imaging carried out 33-34 h APF showed overstretched exuvial Dpy filaments in the qsm mutant, which were not observed in the control (Figures 3C–3F). These results suggest that the resilience of exuvial Dpy filaments is reduced in the qsm mutant, causing the adhesion of the tendon epidermis to the pupal cuticle to fail under the tension from the developing flight muscles.

Qsm and Dpy show different extracellular distribution

In addition to the extracellular localization, Dpy-YFP in tendon cells was found as rapidly moving puncta co-labeled with membrane marker mCherry-CAAX (Figures 4A–A’; Video S2). Dpy-YFP also colocalized with the mCherry-Qsm knockin protein (Figures 4B–B’; Video S3), suggesting that those ZPD proteins are co-produced and co-transported in the same vesicles that reach the interface of tendon cell and extracellular Dpy filaments. In the exuvial space, mCherry-Qsm was diffusely distributed without any distinct accumulation (Figure 4C). The liquid present in the exuvial space can be retrieved as molting fluid from pupae. Molting fluid from a dpy-YFP, mCherry-qsm pupa contained abundant mCherry-Qsm fluorescence, but no Dpy-YFP was detected (Figures 4D and 4D’). To further characterize the dynamics of the extracellular Dpy and Qsm, fluorescence recovery after photobleaching (FRAP) experiment was performed on dpy-YFP, mCherry-qsm pupa at 34 h APF (Figures 4F, 4F’, 4G, 4G’, 4H).
Fluorescence of cuticular and exuvial Dpy-YFP disappeared immediately after 20 s of photobleaching, and remained low-level 2 min later, suggesting that the mobility of extracellular Dpy-YFP is slow (Figures 4F’, 4G’, 4H’, and 4J), as previously shown.20 Conversely, mCherry-Qsm fluorescence in the exuvial space was already present at the time of imaging in the condition where its cytoplasmic fluorescence was eliminated, and was fully recovered 2 min later (Figure 4F–4H and 4I). These results suggested that extracellular mCherry-Qsm is mainly present in the mobile form, while Dpy-YFP is present in an immobile form.

Homozygotes of mCherry-Qsm knockin allele were morphologically normal, suggesting that mCherry-Qsm protein has the normal qsm function when expressed in its native expression domain. mCherry-Qsm expressed in the muscle by Mef2-Gal4 accumulated in the body cavity but not in the exuvial space at a significant level (Figure S4A). We noted numerous vesicles containing mCherry-Qsm inside the tendon cells. Those vesicles also contained Dpy-YFP, and moved toward the apical surface, suggesting that mCherry-Qsm is taken up by tendon cells and partitioned to the Dpy secretion pathway (Figures S4B and S4C; Video S4).

We tested mCherry-Qsm in rescue experiments with sr-Gal4 and pnr-Gal4, and found that the comma and vortex phenotypes were rescued but not the oblique wing phenotype (Figures S2C, S2C’, and S2I). A mutant form of mCherry-Qsm, in which Gly

Figure 2. Genetic interaction of qsm and dpy

(A) Diagram of the Qsm protein drawn by IBS.37 Bold font indicates signal peptide. Five bp deletion in qsmF17fs introduced a frameshift of Qsm open reading frame at F17. Vertical red line next to ZP domain is the consensus furin cleavage site (CFCS). ZP domain: Zona pellucida domain; CC: Coiled-coil domain; TMD: transmembrane domain.

(B–H) The adult phenotypes of various qsm and dpy mutant combinations. See Figure S2 for additional results. Note that sr-Gal4 expressed in the tendon cells (see Figure S3) rescued not only the notum phenotype but also the wing phenotype of qsm (F).

(I) Release of the muscle-tendon tension by tendon cell-specific Drok knockdown suppressed the notum phenotype of qsmF17fs, but not the wing phenotype. (H) Drok knockdown in the tendon cells did not cause defects in the external appearance.

(I) Frequency of the phenotypes. Scale bar, 500 μm (B–H); 200 μm (B’–H’).
residues replaced eight Cys residues, driven by sr-Gal4 or pnr-Gal4 failed to rescue the qsm phenotypes (Figures S2D, S2D', and S2I), suggesting the ZPD is critical to the Qsm function. However, Mef2-Gal4 driven mCherry-Qsm failed to rescue any of the qsm phenotypes (Figures S2G, S2G', and S2I). The difference in the rescue efficiency of untagged Qsm and mCherry-Qsm indicates that mCherry fusion compromised some aspects of the Qsm function.

Interaction of Dpy-ZPD and Qsm
To address the molecular basis of the Dpy-Qsm interaction, we focused on the ZPD of Dpy. Thus, we created several UAS-based constructs that allowed us to overexpress tagged proteins for biochemical assays. To overcome the difficulty of handling the giant Dpy protein, we used the minimal Dpy construct, mVenus-Dpy-ZPD, which contains a signal peptide, mVenus, and the C-terminal region of Dpy, including the ZPD, transmembrane domain, and cytoplasmic region. Immunoprecipitation of mCherry-Qsm from the cell extract allowed the recovery of the long form of mVenus-Dpy-ZPD with intact transmembrane domain and the C terminus (form D1; Figure 5Af) but not the Furin cleaved short form D2. The mCherry-Qsmmut, in which eight Cys residues in ZPD were replaced with Gly residues, still interacted with mVenus-Dpy-ZPD, suggesting that complex formation can

Figure 3. Qsm is essential for Dpy filaments strength
(A and B) FV1000 time-lapse imaging of Dpy-YFP (green) and tendon cells (magenta) in control and qsm−/− pupae (see also Video S1). Scale bar, 20 μm. (C and D) Airyscan images at 33–34 h APF. C: pupal cuticle; E: exuvial space. Scale bar: 10 μm. (E-F) Enlarged view of Dpy filaments. Note discontinuity of YPF fluorescence in some parts of the filaments (arrows) that become more widely spaced in qsm the mutant. (G and H) Measurement of Dpy filaments in the exuvial space and tendon cell length.
Figure 4. Localization of Qsm and Dpy

(A) Dpy-YFP localizes in intracellular vesicles of tendon cells (see also Video S2). Scale bar, 10 μm.

(B) Dpy-YFP and mCherry-Qsm colocalize in intracellular vesicles of tendon cells (see also Video S3, Figure S4, and Video S4 for additional results). Scale bar, 10 μm.

(C) Dpy-YFP and mCherry-Qsm show distinct but overlapping localization patterns in the exuvial space. The mCherry-Qsm signal was enhanced compared to (B). Pupal cuticle (c); exuvial space (e). Scale bar, 10 μm.

(D) Molting fluid isolated from the 3-day old Dpy-YFP, mCherry-Qsm pupa contained a high level of mCherry-Qsm, but no Dpy-YFP above background level was observed. Scale bar, 50 μm.
Role of Qsm on the matrix property of Dpy

dpy and qsm are also expressed in the larval stages (modENCODE,42 flybase.org). We found that Dpy-YFP concentrated at the larval muscle insertion site of wandering stage larvae where the tendon cell-derived Dpy filaments insert into the larval cuticle (Figures 6A–6G; Video S5) and overlap with mCherry-Qsm (Figures 6H–6J). Dpy filaments were organized in parallel to microtubule arrays of the tendon cells at the tendon cell-cuticle interface (Figures 6D–6F). In the Hyvoltage image, Dpy-YFP signal was detected as distinct spots along filaments with regular spacing (Figure 6D). Chitin signal was reduced in this region compared to other cuticles (Figures 6B and 6C; Video S5), and the remaining chitin signal was detected interdigitating with Dpy-YFP (Figures 6D, 6D', 6D'', and 6D'''). In the qsmF17mut mutant, the amount of Dpy-YFP in the muscle insertion sites was reduced to ~70% of the control level (Figures 6L–6N), suggesting that the full-level assembly of Dpy filaments requires Qsm. A similar but more irregular pattern of discontinuous YFP fluorescence was observed in the pupal Dpy-YFP in the exuvial space (Figure 3E), and the distance between the Dpy-YFP spot was increased in the qsm mutant (Figure 3F).

DISCUSSION

At each stage of insect molt, epidermal morphogenesis takes place under the condition of their apical side being covered with apical ECM. Dpy is a major apical ECM component in the exuvial space required for proper morphogenesis of the adult notum, wing,21,22 male genitalia,43 and the embryonic trachea.19,20 Dpy distribution is non-uniform due to localized transcription44 and sometimes forms long strands connecting specific sites of the tissue and pupal cuticles.45 Here we showed that Dpy forms filamentous structures that connect the apical surface of tendon cells to the cuticle. We propose that Dpy filaments support the notum epidermis from collapsing under the strong pulling force from the developing indirect flight muscles.

In the tendon structure of Dpy-YFP larvae, the YFP signal is arranged in a periodic pattern in parallel arrays of filaments, indicating that Dpy forms filaments by ordered association. The interval of YFP signal (0.55 μm) is significantly shorter than the length of Dpy polypeptide in a fully stretched form (3-8 μm, based on predicted Dpy length and the peptide bond length of 0.35 nm in beta-sheet configuration), suggesting that the Dpy protein is folded and assembled into a compact structure, and this may contribute to the tensile property of the filament. Furthermore, Dpy filaments are separated by a space of 0.67 μm that is filled with chitin. Those protein and polysaccharide complex would collectively stabilize the mature tendon matrix, the role previously ascribed to tonofilaments. However, the nearly uniform pattern of Dpy filaments filling the muscle insertion site appears different from the image of tonofilaments observed in late embryos that show a few distinct filaments under the transmission electron microscope.11 Whether Dpy is a structural component of tonofilaments remains to be evaluated.

Dpy matrix attached to tendon cells increased in amount (Figure 3A) and acquired filamentous organization under tension. The filamentous conversion of the Dpy matrix may involve tension-induced Dpy-matrix stretching and assembly of newly secreted Dpy into filaments. It is known that soluble Fibronectin (FN) dimers are converted into fibril under cytoskeletal tension applied to the FN receptor integrin,45–48 the tension-dependent FN remodeling phenomenon was also observed in Xenopus and zebrafish tissues.49,50
We identified Qsm as a factor required for Dpy remodeling. In normal qsm−/− pupae, Dpy-YFP filaments spanning the exuvial space opened up to ~30 μm. Without qsm, Dpy filaments were overstretched at the high tension status (32–34 h APF), opening the exuvial space up to 3-fold. Therefore, we propose that muscle-derived tension and Qsm-dependent modulation are the major sources of mechanical and chemical effectors of Dpy matrix maturation, respectively.

Given the critical requirement for Dpy matrix rigidity and the presence of ZPD, a signature of structural protein, the diffusible nature of Qsm in the exuvial space was unexpected. Here, we discussed the potential action of Qsm in the protein secretory pathway of Dpy.

To study the properties of ZPDs, we used a mini-construct mVenus-Dpy-ZPD, which was cleaved to release ZPD that was secreted and formed oligomers in cell culture. The association of Qsm with the membrane-bound form of Dpy-ZPD inside the cell was consistent with the observation of co-transportation of Dpy and Qsm in the tendon cells. In contrast, we detected only a minimal level of complex formation by extracellular Dpy-ZPD and Qsm. These data suggest that the interaction between Dpy-ZPD and Qsm occurs as immature membrane-bound forms prior to the formation of proper disulfide bonds. The complex of Dpy-ZPD and Qsmmut may stall as maturation intermediates and are degraded as misfolded proteins. In this respect, Qsm functions as a specific co-factor, a molecular chaperon, and is likely important for the conformational maturation of Dpy-ZPD.

We propose a possible mechanism based on our in vitro assay. After Qsm and Dpy-ZPD acquire the proper conformation and disulfide bonds, they dissociate from each other, separate from the membrane following the Furin cleavage, and are delivered to the protein secretory pathway. Dissociation of the Dpy-Qsm complex after this stage is probably important for Dpy maturation since the excessive expression of Qsm was inhibitory to the oligomerization of Dpy-ZPD. As the majority of Dpy consists of a large number of EGF repeats, Dpy maturation and secretion may involve additional mechanisms. Proper deposition and maturation of this gigantic protein is complex, involving regulation at the ER exit site by Tango1, glycosylation and perhaps many other regulators. Another example of ZPD proteins that require each other for their normal secretion is NOAH-1 and NOAH-2 in C. elegans, which are the apical ECM proteins required for epidermal integrity and muscle anchoring. In qsm mutants, expressed Qsm rescued defects in distant locations, suggesting that secreted Qsm diffuses in the exuvial space and interacts with the target tissues to support proper Dpy filament formation. We observed that tendon cells incorporate mCherry-Qsm expressed in an ectopic tissue (muscle) and package into cellular vesicles containing Dpy-YFP that are transported to the interface of tendon cells and apical-ECM (Figure S4; Video S4). This delivery process may involve endocytosis and retrograde recycling through the ER-Golgi compartment, as previously shown for the secreted enzyme chitin deacetylase Serpine1 in the remodeling of the tracheal apical ECM. Dpy-YFP co-secreted with mCherry-Qsm may be incorporated into Dpy filaments by the secretion and capture mechanism. Diffusing Qsm would be incorporated by distant tissues and regulate the Dpy maturation and secretion. In addition, diffused Qsm may directly interact with extracellular Dpy to promote the remodeling of the Dpy matrix, although extracellular Dpy-Qsm interaction remains to be established. Fusion of mCherry somewhat compromised the long-range rescue activity of Qsm. This may be due to the reduction of the diffusion constant, or the mCherry moiety interfering with retrograde transport.

Qsm was first identified based on the screening for circadian regulated genes. Subsequent studies revealed that qsm is expressed in clock neurons in the adult brain in a light-inducible manner. It was hypothesized that Qsm works cell-autonomously to regulate the degradation of the transcription factor circadian. However, how this model fits our finding of Qsm as a soluble extracellular protein and a modulator of Dpy is unknown. Thus, further studies on the behavior of Qsm protein in the extracellular space and intracellular compartment, and

Figure 5. Qsm modulates the stability and polymerization of Dpy-ZPD
(A) Co-immunoprecipitation assay of Qsm Dpy-ZPD interaction in whole-cell extracts. Right: Schematics of expressed proteins and their cleavage products used in the assays. Left: western blots. Input IB-GFP blot shows two forms of mVenus-Dpy-ZPD (D1, D2). Co-expression of mCherry-Qsmmut reduced the amount of mVenus-Dpy-ZPD (Compare lane f and g). Blot of IP-RFP, IB-GFP shows that Qsm associates with the D1 form of mVenus-Dpy but not D2, and this interaction is not compromised by the Cys to Gly residues replacement introduced to mCherry-Qsmmut (lane f and g). Robust association to D1 and D2 was observed for positive control constructs containing Vhh GFP binder (Qv1, Qv2, Qv3, and V1; see lane c). Note that half of UAS-mVenus-Dpy-ZPD was used in lanes f–I compared to lane e to keep the total amounts of UAS plasmids used for each transfection. ZPD: zona pellucida domain; TMD: transmembrane domain; Vhh: VhhGFP4, GFP nanobody
(B) Qsm promotes the secretion of processed Dpy-ZPD. mVenus-Dpy-ZPD in the culture supernatant was detected after immunoprecipitation. A small amount of mVenus-Dpy-ZPD D2 form was detected in the immunoprecipitate of mCherry-Qsm (lane d of IP-RFP, IB-GFP). The D2 form of secreted mVenus-Dpy-ZPD was detected in the supernatant (IP-GFP, IB-GFP, lane c) that was enhanced by mCherry-Qsm and reduced by mCherry-Qsmmut (IP-GFP, IB-GFP, lane d, e).
(C) Secretion of Dpy-ZPD oligomer. mVenus-Dpy-ZPD D2 form was recovered by anti-FLAG immunoprecipitation from the supernatant of cells co-expressing 3xFLAG-Dpy-ZPD and mVenus-Dpy-ZPD (lanes h–j). No complex was detected by mixing supernatants of cells individually expressing 3xFLAG-Dpy-ZPD and mVenus-Dpy-ZPD (lane f and g).
(D) Effect of Qsm on secreted Dpy-ZPD and its oligomer formation (see also Figure S3). mVenus-Dpy-ZPD was detected by anti-GFP immunoprecipitation from the supernatant of cells co-expressing 3xFLAG-Dpy-ZPD, mVenus-Dpy-ZPD, and different plasmid amount of mCherry-Qsm or mCherry-Qsmmut. The 1 x, 2 x, and 4 x mCherry-Qsm co-transfection increased the secreted mVenus-Dpy-ZPD level. Although the 1 x and 2 x mCherry-Qsm co-transfection increased the level of 3xFLAG-Dpy-ZPD/mVenus-Dpy-ZPD complex formation, the 4 x mCherry-Qsm shows an inhibitory effect. A strong inhibition was observed at all levels of mCherry-Qsmmut co-transfection.
(E) Dpy-ZPD complex produced in S2 cells. S2 cells co-expressing 3xFLAG-Dpy-ZPD and mVenus-Dpy-ZPD were fixed, immunostained, and imaged by Airyscan. Upper panels show the focal plane on the top cell surface covered with filamentous extracellular matrix containing 3xFLAG-Dpy-ZPD (magenta) and mVenus-Dpy-ZPD (green). Lower panels show a cell-free area of the coverslip covered with secreted Dpy-ZPD particles. Scale bar, 5 μm.
Figure 6. Dpy is a molecular anchor at the larval muscle insertion sites
(A) Fillet preparation of A3-A4 ventral musculature of 3rd instar larva. Dpy-YFP (green) labels muscle insertion sites, and the muscles were labeled with Phalloidin (magenta). Anterior up. Scale bar, 100 mm.
(B–G) Hyvolution image of the VO5 muscle insertion site (boxed in A). x-y views of one focal planes (B) and y-z section (C) of a larva carrying Dpy-YFP (green) and mCherry-tubulin84B driven by sr-Gal4 (magenta, t), stained with markers for muscle (Dlg, white, m) and chitin (Calcofluor White Stain, blue, c). Dotted vertical line

|          | Peak interval (μm) |
|----------|--------------------|
| Filament | 0.55 +/- 0.10      |
| Spacing  | 0.67 +/- 0.17      |

(H) (legend continued on next page)
additional interaction targets of Qsm will be required to understand the full picture of Qsm function.

In conclusion, we identified Qsm as a novel class of ZPD proteins that acts non-structurally to support the apical ECM maturation under mechanical force. Diffusion of Qsm allows whole-body modification of polymer formation of Dpy and possibly other ZPD proteins. Since the timing of Qsm expression during cuticle formation is tightly regulated under the ecdysis program, Qsm may be an excellent candidate for a systemic modulator of apical ECM at the time of dynamic tissue movement of metamorphosis. Recent studies have uncovered ECM modulation mechanisms by regulated synthesis and secretion, subunit assembly, chemical modification, and proteolytic degradation. Qsm is a new class of molecules regulating apical ECM dynamics by targeting a subset of ZPD proteins and is expected to provide a new direction in the developmental control of ECM dynamics.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

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**AUTHOR CONTRIBUTIONS**

Conceptualization, W.C. and S.H.; Methodology, W.C. and S.H.; Investigation: W.C.; Resources, W.C.; Writing – Original Draft, W.C. and S.H.; Writing – Review and Editing, W.C. and S.H.; Supervision, S.H.; Project Administration, S.H.; Funding Acquisition, W.C. and S.H.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-FLAG M2        | Merck  | Cat# F3165; RRID: AB_259529 |
| Anti-FLAG M2-HRP    | Merck  | Cat# A8592 |
| Anti-GFP pAb-HRP-DirectT | MBL | Cat# 598-2 |
| Anti-RFP pAb-HRP-DirectT | MBL | Cat# PM005-7 |
| Anti-GFP mAb-Magnetic Beads | MBL | Cat# 153-11 |
| Anti-RFP mAb-Magnetic Beads | MBL | Cat# 165-11 |
| Alexa Fluor® 647 Phalloidin | Thermo Fischer | Cat# A22287; RRID: AB_2620155 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| In-Fusion HD Cloning Kit | Takara bio. | Cat# 639634 |
| Paraformaldehyde    | TAAB   | Cat# P001 |
| Takara Ex Taq       | Takara bio. | Cat# RR001A |
| KOD-Plus-Neo        | TOYOBO | Cat# KOD-401 |
| Effectene transfection reagent | QIAGEN | Cat# 301425 |
| Mirus TransIT-insect transfection reagent | Mirus | Cat# MIR 6100 |
| VECTASHIELD antifade mounting medium with DAPI | Vector laboratories | Cat# H-1200; RRID: AB_2336790 |
| VECTASHIELD antifade mounting medium | Vector laboratories | Cat# H-1000; RRID: AB_2336789 |
| 10 x Phosphate Buffered Saline | Nacalai Tesque | Cat# 27575-31 |
| Glass bottomed culture dish (35 mm) | Iwaki Co. Ltd. | Cat# 3910-035 |
| Calcofluor White Stain | Merck | Cat# 18909 |
| Amersham ECL prime western blotting detection reagent | Merck | Cat# GERP2232 |
| Schneider’s Drosophila medium | Thermo Fischer | Cat# 21720024 |
| Fetal bovine serum | Equitech | Cat# SFBM30-0050 |
| Penicillin-Streptomycin | Thermo Fischer | Cat# 15070063 |
| Protease Inhibitor Cocktail | Nacalai Tesque | Cat# 03969-21 |
| Zero Blunt® TOPO® PCR Cloning Kit | Thermo Fischer | Cat# 450245 |
| SuperSep Ace 5-20% | Fujifilm Wako Pure Chemical | Cat# 197-15011; Cat# 194-15021 |
| Experimental Models: Cell lines |        |            |
| Drosophila Schneider 2 cells | RIKEN Bioresource Center | RCB1153; RRID: CVCL_Z232 |
| Experimental Models: Organisms/Strains |        |            |
| D. melanogaster: y,w | A. Nakamura | N/A |
| D. melanogaster: sr-Gal4 (on III) | Bloomington Drosophila Stock Center (BDSC) | RRID: BDSC_26663; FlyBase: FBst0026663 |
| D. melanogaster: sr-Gal4, UAS-mCherry-CAAX (on III) | This work | N/A |
| D. melanogaster: pnr-Gal4, UAS-mCherry-CAAX (on III) | BDSC | RRID: BDSC_3039; FlyBase: FBtl0004011 |
| D. melanogaster: pnr-Gal4, UAS-mCherry-CAAX (on III) | This work | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| D. melanogaster: UAS-mCherry-tub84B (on III) | Atsushi Wada, unpublished. | N/A |
| D. melanogaster: UAS-mCherry-CAAX (on III) | FlyBase: FBtp0041366 | 40 |
| D. melanogaster: UAS-qsm (on III) | FlyBase: FBtp0067752 | 33 |
| D. melanogaster: 1151-Gal4; UAS-mCD::GFP | Ken-ichi Kimura (Hokkaido University of Education) | FlyBase: FBti0007229 |
| D. melanogaster: UAS-Nεcl (on II) | FlyBase: FBal0048200 | 31 |
| D. melanogaster: UAS-Drok-IR-GD (on II) | Vienna Drosophila Resource Center (VDRC) | VDRC: 3793; FlyBase: FBst0462239 |
| D. melanogaster: UAS-Drok-IR-KK (on II) | VDRC | VDRC: 104675; FlyBase: FBst0476533 |
| D. melanogaster: dpy-YFP (on II) | Kyoto Stock Center | RRID: DGGR_115238; FlyBase: FBti0143891 |
| D. melanogaster: mCherry-qsm (knockin) | This work | N/A |
| D. melanogaster: vasa-phiC31; ZH-attP-86Fa | BDSC; 37 | RRID: BDSC_24486 |
| D. melanogaster: y, w; Crey; D/YT3, Sb | BDSC; 64 | RRID: BDSC_851 |
| D. melanogaster: UAS-mCherry-qsm (on III, 86Fa) | This work | N/A |
| D. melanogaster: UAS-mCherry-qsm\textsuperscript{mtd} (on III, 86Fa) | This work | N/A |
| D. melanogaster: dpy-YFP, mCherry-qsm | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176} | This work | N/A |
| D. melanogaster: dpy\textsuperscript{avr}, qsm\textsuperscript{F176} | Kyoto Stock Center | RRID: DGGR_105826; FlyBase: FBal0002834 |
| D. melanogaster: dpy\textsuperscript{avr}, qsm\textsuperscript{F176}, dpy-YFP/ CyO, Dfd-EYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/ CyO, Dfd-EYFP; sr-Gal4/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; sr-Gal4, UAS-mCherry-CAAX/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; sr-Gal4, UAS-mCherry-tub84B/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; sr-Gal4, UAS-mCherry-CAAX/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; sr-Gal4, UAS-mCherry-tub84B/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; sr-Gal4, UAS-mCherry-qsm\textsuperscript{mtd}/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; UAS-qsm/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; UAS-mCherry-qsm/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; UAS-mCherry-qsm\textsuperscript{mtd}/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; UAS-mCherry-qsm/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
| D. melanogaster: qsm\textsuperscript{F176}, dpy-YFP/CyO, Dfd-EYFP; UAS-mCherry-qsm\textsuperscript{mtd}/ TM6B, Dfd-GMR-nvYFP | This work | N/A |
## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| D. melanogaster: qsm^{176}F, dpy-YFP/CyO, | This work | N/A |
| Dfd-EYFP; UAS-kon-IR/ TM6B, | | |
| Dfd-GMR-nvYFP | | |
| D. melanogaster: dpy-YFP, | This work | N/A |
| UAS-nc^{17}F/CyO, Dfd-EYFP | | |
| D. melanogaster: dpy-YFP; sr-Gal4/ | This work | N/A |
| TM6B, Dfd-GMR-nvYFP | | |
| D. melanogaster: dpy-YFP; sr-Gal4, | This work | N/A |
| UAS-mCherry-CAAX/ TM6B, | | |
| Dfd-GMR-nvYFP | | |
| D. melanogaster: dpy-YFP; Mef2-Gal4/ | This work | N/A |
| TM6B, Dfd-GMR-nvYFP | | |
| D. melanogaster: dpy-YFP; Mef2-Gal4, | This work | N/A |
| UAS-mCherry-CAAX/ TM6B, | | |
| Dfd-GMR-nvYFP | | |
| D. melanogaster: dpy-YFP; qsmF176F, | This work | N/A |
| UAS-Drok-IR-KK/ CyO, Dfd-EYFP | | |
| D. melanogaster: Mef2-Gal4 | BDSC | RRID: BDSC_27390; Flybase: FBtp0006434 |
| D. melanogaster: UAS-ChlVis-Tomato | BDSC;^9 | RRID: BDSC_66512; FlyBase: FBst0066512 |
| D. melanogaster: UAS-kon-IR | NIG-Fly | NIG.10275R-1 |
| (perd-IR) | | |
| D. melanogaster: y^{+} | BDSC | RRID: BDSC_40161; FlyBase: FBst0040161 |
| M{vas-int.Dm}ZH-2A w^{+} | | |
| D. melanogaster: y^{2} cho^{2} v^{+} P{nos-Cas9, y+, v+}1A/FM7c, | NIG-Fly | CAS-0002 |
| KrGAL4 UAS-GFP | | |

### Oligonucleotides

| Primer: FLAG-Linker-Intron-Fw1 | "GATTACAAGGACGATGACG ACAAGGCGAGGTAAATA TAAAT" | This work | N/A |
| Primer: FLAG-Dpy-SS-Rv1 | "TCGTCATCGTCCCTTGTA ATCTGAAATGACTG CACTCGA" | This work | N/A |
| Primer: pUASTattB backbone-Fw1 | "TACCAGGTTCTTTGATTAC" | This work | N/A |
| Primer: pUASTattB backbone-Rv1 | "GTAATCGAAAGAACCT GGTA" | This work | N/A |
| Primer: 3xFLAG-1-Fw1 | "GATTACAAGGATCGA CCGCGACTACAAGGAC CAGATATTGATTACAAGG ACGATGAC" | This work | N/A |
| Primer: SS-FLAG-Rv1 | "CCGTCGTGATCCTTGTAAC TCTGAATGTACTGCA" | This work | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pBFv-U6.2           | RRID: Addgene_138400; FlyBase: FBmc0003127 |
| pBFv-U6.2-qsm gRNA   | This work | N/A |
| pUASTattB           | http://www.flyc31.org/ |
| pUASTattB-mCherry-qsm | VectorBuilder: VB181004-1024tea |
| pUASTattB-mCherry-qsm<sup>mut</sup> | VectorBuilder: VB181009-1017pyw |
| pUASTattB-VhhGFP4-mCherry-qsm | VectorBuilder: VB181009-1028nku |
| pUASTattB-VhhGFP4-mCherry | VectorBuilder: VB181009-1030nht |
| pUASTattB-mVenus-Dpy-ZPD | VectorBuilder: VB181129-1469ekk |
| pUASTattB-3xFLAG-Dpy-ZPD | This work | N/A |
| pWA-GAL4            | Yasushi Hiromi (NIG, Japan) FlyBase: FBtp0010842 |

**Software and Algorithms**

- **EDISON-WMW: Exact Wilcoxon-Mann-Whitney Test Calculator** [66](https://ccb-compute2.cs.uni-saarland.de/wtest/)
- **IBS** [67](http://ibs.biocuckoo.org/)
- **ImageJ-Fiji LOCI** [68](https://fiji.sc) RRID: SCR_002285
- **Imaris** Oxford instruments [https://imaris.oxinst.com/](https://imaris.oxinst.com/) RRID: SCR_007370
- **GIMP** GIMP.org [https://www.gimp.org/](https://www.gimp.org/) RRID: SCR_003182
- **Huygens (HyVolution 2)** Scientific Volume Imaging [https://svi.nl/HyVolution](https://svi.nl/HyVolution)
- **Office 365 (Word, Excel, Powerpoint)** Microsoft [https://www.office.com/](https://www.office.com/)
- **OrientationJ Distribution** [30](http://bigwww.epfl.ch/demo/orientation/) RRID: SCR_014796
- **OrientationJ Measure** [69](http://bigwww.epfl.ch/demo/orientation/) RRID: SCR_014796
- **R** [70,71](https://www.r-project.org/) RRID: SCR_001905
- **ROI 1-Click** [72](https://github.com/LauLauThom/Fiji-RoiClickTools)
- **Zen** Carl Zeiss [https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html](https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html) RRID: SCR_018163

**Other**

- **Confocal microscope** Carl Zeiss LSM880 with Airyscan detector
- **Confocal microscope** Olympus FV1000 with GaAsP detector
- **Confocal microscope** Leica TCS SP8X with HyVolution 2
- **Digital Microscope** Keyence VHX-6000
- **Micro needle** Shiga Koncyu Cat# 251; [http://www.shigakon.com/](http://www.shigakon.com/)
- **SILPOT 184 W/C** Dow [https://www.dow.com/en-us/pdp.dowsil-silpot-184-wc.03255981h.html](https://www.dow.com/en-us/pdp.dowsil-silpot-184-wc.03255981h.html)
RESOURCE AVAILABILITY

Lead Contact
Further requests for information, resources, and reagents should be directed to and will be fulfilled by the Lead Contact, Shigeo Hayashi (shigeo.hayashi@riken.jp).

Materials Availability
All unique/stable reagents generated in this study are available with the Lead Contact after signing a Materials Transfer Agreement.

Data and Code Availability
The RAW datasets supporting the current study have not been deposited in a public repository because of the large size of files, but are available from the Lead contact on request. This study did not generate code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Drosophila melanogaster* strains were maintained in the vials with standard yeast-cornmeal-agar media and were raised at 25°C. Female adults were used to showing the representative image of external phenotypes, and both sexes were used for the phenotypes scoring and imaging. For analysis of pupal tissues, white prepupae (APF 0 h) were collected by a soft and wetted brush and cultured at 25°C before imaging. *Drosophila* male Schneider 2 (S2) cells were cultured in the commercial Schneider’s *Drosophila* medium supplement with 10% fetal bovine serum and 100 unit/mL penicillin-streptomycin. Cells were cultured in flasks or multi-well culture plates at 25°C.

METHOD DETAILS

*Drosophila* strain and genetics
All *Drosophila* experiments were conducted at 25°C. The qsm mutant was generated by the CRISPR/Cas9 technique. Complementary DNA oligonucleotides of gRNA sequence (GTGGCTGGCGGCCCTATTCTG) to the first exon of qsm was cloned to the gRNA expression vector pBFv-U6.2. The pBFv-U6.2-qsm gRNA vector (200 ng/μl) was injected to nos-Cas9 embryos (CAS-0002, National Institute of Genetics). The hatched flies were crossed to y w; Gla/Cyo, Dfd-EYFP for two generations. Independent lines were established and maintained over CyO, Dfd-EYFP balancer. The DNA region flanking the gRNA target site was PCR amplified from genomic DNA extracted from single homozygous flies. The PCR products were cloned in the zero blunt TOPO vector for sequencing. A line with 5 bp deletion causing protein truncation (qsmF17fs) was chosen for subsequent experiments. Unlike dpy null alleles that are embryonic lethal, with severe defects in the trachea, qsmF17fs was homozygous, viable, and fertile. This indicates that while qsm is required for dpy function in adult morphogenesis, it is apparently dispensable for the vital dpy function. Although the precise reason is unknown, we speculate that exceptionally high mechanical load applied to adult tissues during metamorphosis may have prompted the requirement for the additional mechanisms.

*mCherry*-qsm knockin line was generated by fC31 recombinase-mediated cassette exchange. *qsm* was crossed to the vas-fC31 integrase expressing strain (BDSC-40161), and the progeny embryos were injected with pBS-KS-attB1-2-PT-SA-SD-1-mCherry (200 ng/μl). The successful cassette exchange event was identified by the loss of y marker, expression of mCherry, and DNA sequencing. These UAS-transgene flies were generated by fC31 recombinase-mediated integration through microinjection of the embryos with fC31 and zh-86Fa landing site. The selection marker 3xP3-RFP on the zh-86Fa of the established independent lines was further removed by crossing with Cre-recombinase expressing flies. Independent lines were established and maintained over TM6B, Dfd-GMR-nvYFP balancer.

Molecular cloning
The pUASTattB-based plasmids were constructed by VectorBuilder Inc. FLAG-tagged Dpy-ZPD was constructed as follows: Two DNA fragments were amplified with KOD Plus Neo (TOYOBO, Japan) using pUAST-attB-mVenus-Dpy-ZPD as the template, with the following primer pairs: FLAG-Linker-Intron-Fw1 “GATTACAAGGCGATGACGACAAAGGGCGAGGTAAATATAAAT” and pUASTattB backbone-Fw1 “TACCAGTTCTTTTGATTAC”; FLAG-Dpy-SS-Rv1 “TCGTCATCGTCCTTTGAATCTCTGAATGTACTGCACCTGA” and pUASTattB backbone-Rv1 “CGTCGTGATCCTTGTAATCTCTGAATGTACTGCA.” These two amplified DNA fragments were fused using In-Fusion HD Cloning Kit, resulting in an intermediated construct pUASTattB-FLAG-Dpy-ZPD. This construct was used as a template to amplify two DNA fragments with the following primer pairs: pUASTattB backbone-Rv1 “GTAATCGAAAAGACCTGGTA” and 3xFLAG-Fw1 “GATTACAAGGATGACGAGGCGACTACAGGACCCAGATATTGATTTACAAGGACGATGAC”; pUASTattB backbone-Fw1 “TACCAGTTCTTTTGATTAC” and SS-FLAG-Rv1 “CGTCGTGATCCTTTGAATCTCTGAATGTACTGCA.” Subsequently, the resulting DNA fragments were fused to produce pUASTattB-3xFLAG-Dpy-ZPD. The constructs were confirmed by restriction enzyme digestion and sequencing.
Immunofluorescence and antibodies
Flat preparations of larval body wall were carried out as follows: Wandering 3rd instar larvae were washed in phosphate-buffered saline (PBS), pinned with microneedles onto the stage of silicon rubber (SILPOT 184) solidified in the 60 mm plastic dish, cut along the dorsal midline with microsurgical scissors, and the body wall was stretched open with microneedles. Tissues were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After the gut and fat body were removed and stained with Calcofluor to label chitin, tissues were cut to appropriate sizes and mounted with Vectorshield.

S2 cells grown on 12 mm round coverslip in the 35mm culture dishes were transfected. After 48 h, coverslips were picked up and placed on parafilm. Fixation with 4% paraformaldehyde in PBS, washing with 2% bovine serum albumin (BSA), 0.2% of Triton X-100 in PBS, and antibody incubation were all performed on parafilm in the plastic box. The coverslips with the stained S2 cells were mounted to glass slides with Vectorshield.

Fluorescence microscopy
Images from the fixed larval samples were taken using an upright Olympus FV1000 with UPLSAPO 10X2 NA-0.4 and UPLSAPO60XW 60x NA-1.2 water-immersion lenses. High-resolution images of larval samples were acquired using Leica TCSP8X with Hyvolution2, by using HC PL APO 63x OIL CS2 oil-immersion (NA-1.4) lens. Time-lapse live images of pupal samples were acquired using an inverted Olympus FV1000 equipped with a UPLSAPO 30X NA 1.05 silicone oil-immersion lens and GaAsP detectors. The sample mounting method for long time pupal live imaging was a modified version of one previously reported. Briefly, notums were exposed by partially removing the pupal case and placed dorsal side down on the glass-bottom dish by fixing with small pieces of double-stick tape. Water or 50% glycerol was used as the mounting medium. Images of pupal samples were acquired using Zeiss LSM880 with Airyscan detector, equipped with a C-Apochromat 40x/1.2 W Corr M27 or Plan Apochromat 63x SR Oil-immersion lenses. Pupae removed from all pupal cases were mounted on the glass slides as described previously. Instead of the moist Whatman paper, we used two small pieces of wet tissue paper to prevent dehydration. Images were processed using ImageJ-Fiji and GIMP.

Plasmids transfection
*Drosophila* S2 cells were cultured one day before transfection. The plasmids were transfected using Effectene (QIAGEN) or Insect Transit transfection reagent (Mirus). After transfection, cells were grown at 25°C for 48 h before imaging or isolation of the cell pellet and media, which were isolated by 114 g centrifugation. Cell pellets were washed using 1 × PBS solution. The pellets and media were then stored at –80°C before use.

Fluorescence recovery after photobleaching (FRAP)
The pupa samples were mounted as described above. FRAP was performed using Zeiss LSM-880 with Airyscan confocal microscope. The region of interest (ROI) was defined using the Zeiss Zen software. Full lasers power was used for bleaching the ROI for 20 s. The confocal images were acquired before the bleaching, immediately and 2 min after the bleaching.

Immunoprecipitation and western blotting
Immunoprecipitation experiments were performed according to the manual of magnetic beads (MBL) with some modifications. Briefly, 1 to 1.5 mL of culture supernatant was mixed with protease inhibitors cocktail (Nacalai Tesque), followed by centrifugation at 16,000 g for 20 min at 4°C to remove cell debris. Whole-cell lysates were made by thawing cell pellets suspended with 30 μL of cold lysis buffer (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40) containing the protease inhibitor cocktail (Nacalai Tesque). Samples were incubated for 30 min with gentle agitation at 4°C and then centrifuged at 16,000 g for 20 min at 4°C to remove the cell debris. Each sample was mixed with 20 μL of Anti-GFP mAB-Magnetic Beads or Anti-RFP mAB-Magnetic Beads (MBL) and incubated with gentle agitation for 1 h at 4°C. The beads were then washed with IP wash buffer (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.05% NP-40) four times with a magnetic rack. Immunoprecipitates of magnetic beads were re-suspended in 20 μL of Laemmli sample buffer and incubated at 95°C for 5 min, and the tubes were placed on the magnetic rack to separate the beads. Samples were loaded onto Wako SuperSep Ace Gel (5%~20%), run at 20 mA for 1 h, and then transferred to the PVDF membranes using iBlot2 Dry Blotting System (Thermo Fisher).

Western blotting was done using the iBind Flex Western Device (Thermo Fisher), following the manual. The following antibodies were used for the blotting: anti-GFP pAb-HRP-DirectT (MBL) in the dilution of 1:1000 to 1:2000, anti-RFP pAb-HRP-DirectT (MBL) in the dilution of 1:1000 to 1:2000, and Anti-FLAG M2 (Merk) in the dilution of 1:1000. Amersham ECL prime western blotting detection reagent (Merk) was used for signal detection, and the signal intensity of the ROI was marked and measured using an ImageJ plug-in ROI 1-click.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

An ImageJ plug-in OrientationJ Distribution was used for quantifying the exuvial Dpy-YFP orientation distribution. Briefly, the 6 slices of Z-projection images were made by ImageJ-FIJI, and multiple 64 × 64 pixel ROIs of the Dpy-YFP signals were then applied to the “OrientationJ Distribution” using the default settings. The same series of ROI images were used for measure the Dpy-YFP signal coherency by using “OrientationJ Measure”. The data of Dpy-YFP coherency and orientation distribution data were converted into the boxplot and wind rose chart by Excel. The normal distribution of the coherency datasets was verified by the Shapiro-Wilk test.
Analysis of Variance (ANOVA) and two-tailed Student’s t test were employed to calculate the p value by Excel. The number of n indicates the total numbers of ROIs from multiple samples.

To analysis the periodicity of Dpy-YFP Z stack images of a muscle insertion site was captured with Leica TCS-SP8X with HyVo-lution2 (Figure 6B). Line scan of the Dpy-YFP channel was taken from the selected z slice, and each line was analyzed by the Auto-correlation Function (acf) in the R package that detects periodicity. The location of the first peaks of the acf plots was identified as a major interval for each series. The average interval was calculated from multiple line scans.

To compare Dpy-YFP signals between control and qsm mutant (Figures 6L and 6M), maximal intensity Z-projection images were generated by ImageJ-Fiji. The signal intensity of each region was marked and measured manually. The number of n indicates the total number of muscle insertion sites from multiple samples. We used EDISON-WMW, a Wilcoxon-Mann-Whitney test calculator to obtain the p value. Asterisks indicate statistical significance (*)p < 0.05, (**)p < 0.01, and (***)p < 0.001).