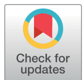
PNAS RESEARCH ARTICLE | DEVELOPMENTAL BIOLOGY



Chitin deacetylases are necessary for insect femur muscle attachment and mobility

Seulgi Mun[a,1], Mi Young Noh[b,1], Erika R. Geisbrecht[c], Karl J. Kramer[c], Subbaratnam Muthukrishnan[c,2], and Yasuyuki Arakane[a,2]

Edited by Lynn Riddiford, University of Washington, Friday Harbor, WA; received November 16, 2021; accepted March 28, 2022

Muscle attachment sites (MASs, apodemes) in insects and other arthropods involve specialized epithelial cells, called tendon cells or tenocytes, that adhere to apical extracellular matrices containing chitin. Here, we have uncovered a function for chitin deacetylases (CDAs) in arthropod locomotion and muscle attachment using a double-stranded RNA-mediated gene-silencing approach targeted toward specific CDA isoforms in the red flour beetle, *Tribolium castaneum* (*Tc*). Depletion of TcCDA1 or the alternatively spliced TcCDA2 isoform, TcCDA2a, resulted in internal tendon cuticle breakage at the femur–tibia joint, muscle detachment from both internal and external tendon cells, and defective locomotion. TcCDA deficiency did not affect early muscle development and myofiber growth toward the cuticular MASs but instead resulted in aborted microtubule development, loss of hemiadherens junctions, and abnormal morphology of tendon cells, all features consistent with a loss of tension within and between cells. Moreover, simultaneous depletion of TcCDA1 or TcCDA2a and the zona pellucida domain protein, TcDumpy, prevented the internal tendon cuticle break, further supporting a role for force-dependent interactions between muscle and tendon cells. We propose that in *T. castaneum*, the absence of *N*-acetylglucosamine deacetylation within chitin leads to a loss of microtubule organization and reduced membrane contacts at MASs in the femur, which adversely affect musculoskeletal connectivity, force transmission, and physical mobility.

tendon | microtubule | locomotion | extracellular matrix (ECM) | zona pellucida domain (ZPD)

Significance

We show that interfering with insect chitin deacetylation by down-regulation of specific chitin deacetylase (CDA) isoforms, belonging to subfamily group I, causes breakage of the chitinous internal tendon cuticle at the femur–tibia joint, muscle detachment from both internal and external tendon cells, and defective locomotion. Our studies reveal a previously unrecognized role of CDA-like proteins in cooperation with zona pellucida domain-containing proteins in musculoskeletal connectivity, maintenance of tendon cell microtubule integrity, muscle force transmission, limb movement, and locomotion. We propose an essential function for group I CDAs, which are highly conserved among insect and other arthropod species, in invertebrate musculoskeletal connectivity involving partially deacetylated chitin in the extracellular matrix overlying the tendon cells.

The stable and force-resistant attachment of muscles via tendons to endo- or exoskeletons (bone or cuticle) is essential for efficient locomotion and for maintenance of the structural integrity of myotendinous junctions (MTJs) in both mammals and arthropods (1–4). At muscle attachment sites (MASs, apodemes) of insects, and arthropods in general, the apical plasma membrane of specialized epithelial cells—variously called apodemes, tendon cells, or tenocytes—adhere to the chitinous extracellular matrix (ECM) and to muscle cells through their basement membrane. At direct attachment sites, where tendon cells attach to single muscles, the hemiadherens junctions (HAJs) that connect them are only about 30- to 40-nm thick, with only a thin layer of electron-dense material in between (5). However, at indirect MASs, the tendon cells have a prominent basal matrix that can be several microns thick and contain other proteins that participate in adhesion to muscle cells (5). It has been proposed that the apical membranes of these cells contain transmembrane proteins with zona pellucida domains (ZPD) that project into the cuticle. These ZPD proteins also possess cytosolic tails that interact with tendon cell constituents (5). On the basal side, the tendon cells attach to muscle cells via electron-dense MTJs where microtubules, integrins, and cytoskeletal actin filaments attach (6, 7).

Previous studies have revealed that the extracellular domains of membrane-bound, cell-specific integrins and their ligands are required for the adhesion of muscle and tendon cells (8–10). In insects, this adhesion is aided by ECM proteins, such as thrombospondin, tiggrins, and laminins that directly interact with integrins, as well as by other adhesive and regulatory proteins secreted into the common ECM shared by these two cell types. *Drosophila melanogaster myospheroid* (*mys*) mutants that lack the β-subunit of the position-specific integrin (11) exhibit detached muscles and dramatically shortened and spherical microtubules and F-actin arrays (9, 12). Through their intracellular domains, the integrins further interact with large intracellular talin proteins. Specific domains of talins associate with cytoskeletal actin and vinculin, and thus are capable of conveying vectorially intracellular mechanosensory inputs from muscle cells to membrane-bound integrins and then to the connected tendon cells. This chain of interactions enables reciprocal signaling between the ECM and the internal contractile machinery (13–15).

Besides efficiently transmitting the force of muscle contraction to the insect's endo- or exoskeleton, these adhesion joints must withstand the repeated and sustained stress

Author contributions: S. Muthukrishnan and Y.A. designed research; S. Mun and M.Y.N. performed research; S. Mun, M.Y.N., E.R.G., K.J.K., S. Muthukrishnan, and Y.A. analyzed data; and S. Muthukrishnan and Y.A. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Copyright © 2022 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

[1]S. Mun and M.Y.N. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: smk@ksu.edu or arakane@chonnam.ac.kr.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2120853119/-/DCSupplemental.

Published June 8, 2022.

experienced during MTJ development and adult locomotion (1, 2, 10, 16). Signaling between muscle cells and tendon cells mediated by secreted ligands and their cognate receptors on cell membranes regulates muscle migration. However, the role of the components of insect tendon cells per se and of the chitinous/collagenous ECM has not been studied as extensively, except for a few studies involving apical membrane proteins that contain ZPDs (5, 17, 18).

Insect chitin deacetylase (CDA)-like proteins have been classified into five groups based upon their domain organization, phylogenetic analyses, and tissue-specific expression (19–21). Group I CDA-like proteins contain a chitin-binding domain, CBM14, a low-density lipoprotein receptor A domain, and a carbohydrate esterase domain, CE4 (www.cazy.org). CDAs (EC 3.5.1.41) are metalloenzymes that catalyze the partial conversion (5 to 20% of the monomeric building block, *N*-acetylglucosamine) of chitin in insect cuticles to partially deacetylated chitin by removing some of the acetyl groups. CDAs are thought to be secreted into the extracellular space and to bring about partial deacetylation of nascent chitin chains (perhaps in association with other proteins) as they are extruded from the cell membrane into the assembly zone (22–24). Of functional significance, CDAs in insects have been shown previously to be essential for maintaining the structural integrity of the laminar cuticle and vertical pore canals (25–28). Down-regulation of transcripts for two *Tribolium castaneum* (*Tc*, red flour beetle) proteins, TcCDA1 or TcCDA2 (both belonging to group I), by injection of gene- or exon-specific double-stranded RNA (dsRNA), resulted in molting arrest and in pupal lethality (Fig. 1*A*) (23). Transmission electron microscopy (TEM) revealed that there was a loss of cuticle integrity, including malformed pore canals and a lack of laminar organization (25). In another beetle species, *Holotrichia parallela* (*Hp*), cuticle organization and chitin laminae were disrupted upon down-regulation of either *HpCDA1* or *HpCDA2* (29). In *Locusta migratoria*, the reduction of CDA levels also enhanced its sensitivity to organophosphorus pesticides and fungal infection (30). Because CDAs are essential for insect cuticle structure differentiation, prevention of xenobiotics penetration and other developmental processes, they have been proposed as molecular targets of minimal-risk green biopesticides (31).

In this report, we have used the red flour beetle, *T. castaneum*, as a model coleopteran insect species (32) to investigate the interaction between chitinous cuticle and tendon cell apical HAJs with attached microtubule arrays and filamentous extensions that penetrate the cuticle, as well as their influence on insect locomotion. Our findings reveal previously unrecognized roles of CDA-like proteins, in association with transmembrane proteins with ZPDs, in the maintenance of tendon cell microtubule integrity, muscle force transmission, and locomotion. Our results suggest a role for partially deacetylated cuticular chitin associated with both external and internal tendon cells (ETCs and ITCs, respectively) in musculoskeletal connectivity and locomotion, possibly by affecting cross-linking with cuticular proteins including ZPD proteins.

Results

CDA Deficiency Leads to Internal Tendon Cuticle Breakage and Defective Locomotion. We have carried out RNA interference (RNAi) experiments using sublethal doses of dsRNAs for *TcCda1*, ds*TcCda1*, or higher doses of the alternatively spliced forms of *TcCda2*, ds*TcCda2a*, and ds*TcCda2b*, which allowed survival to adulthood. Whereas all three sets of dsRNA-treated insects displayed abnormal soft as well as hard cuticles at multiple anatomical locations (25), administration of ds*TcCda1* or ds*TcCda2a* but not ds*TcCda2b* resulted in adult insects with defective limb movement and locomotion (*SI Appendix*, Fig. S1 for the specificity and effectiveness of RNAi at 200 ng and 1 ng dsRNA per insect). In particular, the adults were unable to move their femur–tibia joints, as revealed by the abnormal folding of leg segments (Fig. 1*A* and Movie S1). Insects depleted of the *TcCda2b* splice transcript alone (200 ng dsRNA per insect) did not have any defective locomotion but displayed wrinkled elytra as reported earlier (23, 25). To investigate the cause of the defective leg movement following depletion of TcCDA1 or TcCDA2a, we examined longitudinal sections of the femur–tibia joints from day 5 pupae following *TcCda* RNAi or a control *Vermilion* RNAi (ds*TcVer*-injected; the *Ver* gene affects only eye-color). We used a FITC-labeled chitin-binding protein (FITC-CBD) probe that is specific for chitin to unequivocally establish that both the external and the internal "tendon cells" that constitute MASs (apodemes) secrete a chitinous cuticle (Fig. 1*B*; *SI Appendix*, Fig. S2 for further confirmation of the laminar nature of this chitinous matrix associated with the ITC). The long internal tendon cuticle along with the adhering string of ITCs and their basal matrices constitute the "apodemes" that serve as muscle attachment points. This chitinous internal cuticle, along with the adhering ITCs (apodemes), acting together provide structural support for the appendages and resistivity that counters the forces of muscle contraction. The long internal tendons, sometimes referred to as "internal apodemes," are deposited by appendage-invaginating epithelial tendon precursor cells (33, 34). In *D. melanogaster*, all cells lining the cavity created by tendon cells that invaginate leg segments have been shown to express the transcription factor Stripe (33, 34), considered a hallmark of specialized epithelial cells associated with MASs in the exoskeleton. We will, therefore, refer to the cells attached to the chitinous cuticle inside leg segments as "internal tendon cells" and the cells attached to the exoskeletal chitinous cuticle as "external tendon cells."

In control insects, the internal tendon cuticle junction between the tibial and femur leg segments was intact and showed no breaks (white arrows in Fig. 1*B*). RNAi for *TcCda1* and *TcCda2a*, as well as a combination of these two dsRNAs but not for *TcCda2b* alone, revealed a separation of the chitin-containing internal tendon cuticles of the femur and tibia (Fig. 1*B*), providing a possible explanation for the beetles' observed defective mobility. The tips of the broken cuticles were swollen and retracted from the tibia–femur joint, leaving a large gap between the internal cuticles of the two leg segments (pairs of yellow arrows in Fig. 1*B*). Immunostaining with TcCDA1 or TcCDA2 antibody revealed that these proteins in control insects were localized near the apical membrane of both the ITCs and the ETCs underlying the endocuticle as a narrow band, suggestive of membrane association and localization in the assembly zone of the cuticle (*SI Appendix*, Fig. S3, *Upper*). These results indicated active expression of TcCDA1 and TcCDA2 proteins in ITCs underlying the cuticle near the site where the femur and tibial cuticles connect. Immunostaining following RNAi for *TcCda1* or *TcCda2* revealed that besides depleting the targeted transcripts, the corresponding CDA proteins were also severely depleted in all cuticle-secreting cells, confirming the specificity of the CDA antibodies and also the effectiveness of RNAi even at the protein level (*SI Appendix*, Fig. S3, compare *Upper* panels with *Lower* panels).

CDA Deficiency Causes Muscle Detachment and Loss of Structural Integrity. Labeling for actin in sections of leg segments from control insects at the pharate adult stage using Alexa Fluor 546-labeled phalloidin (stained red) showed stretched femur muscles, in which one end was firmly anchored to the ITC

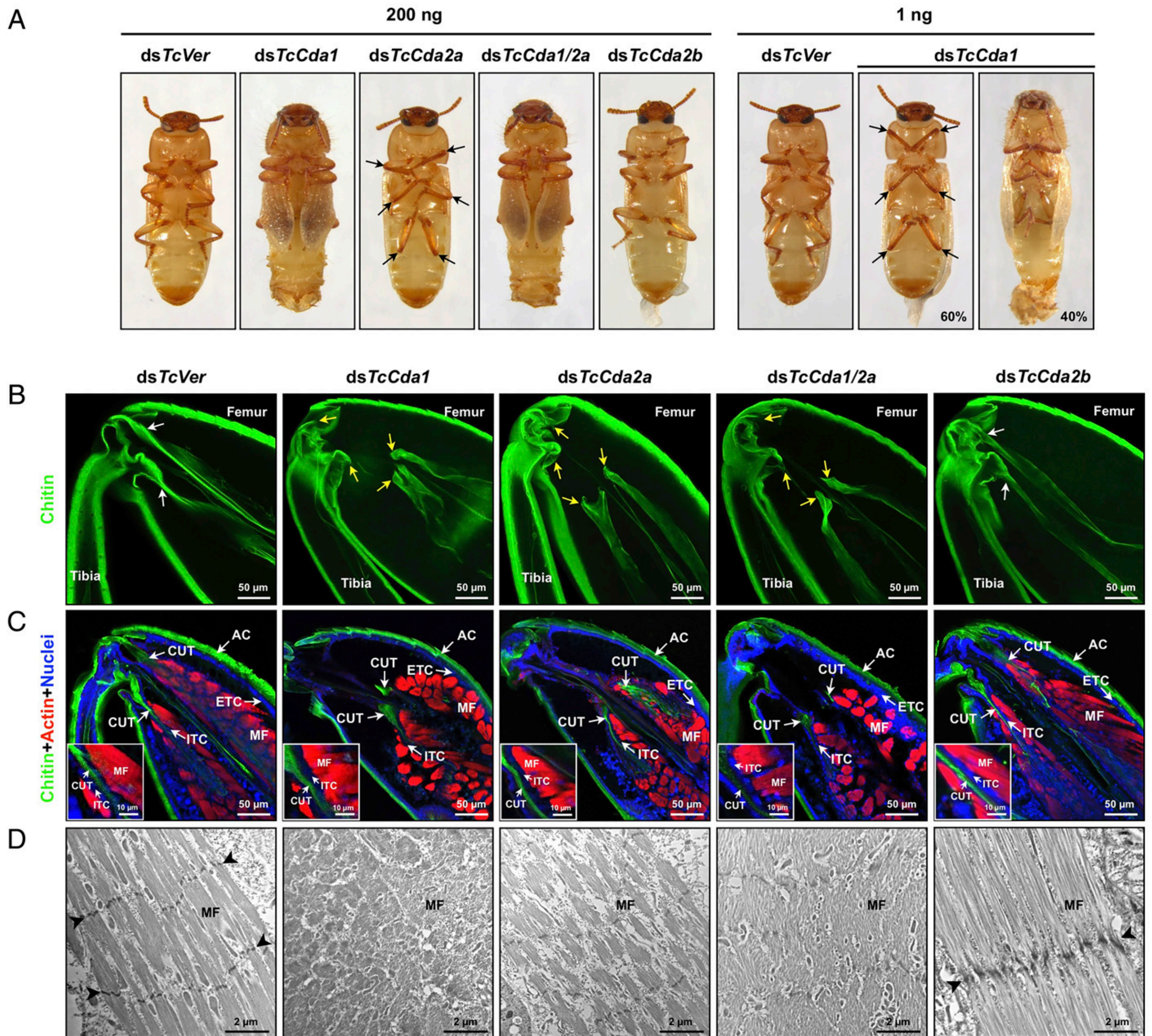


Fig. 1. Morphology of insects, limb tendons and muscles of pharate adults after RNAi for *TcCdas*. dsRNA (200 ng per insect) for *TcVer*, *TcCda1*, *TcCda2a*, *TcCda1/2a*, or *TcCda2b* was injected into day 0 pupae (*n* = 40). (*A*) ds*TcCda1*- or ds*TcCda1/2a*-treated pupae failed to complete adult eclosion and died without shedding their pupal exuviae, while ds*TcCda2a*- and ds*TcCda2b*-treated pupae did molt to adults. However, the resulting ds*TcCda2a*-adults, but not ds*TcCda2b*-adults, were unable to move their femur–tibia joints (indicated by arrows). Similarly, ~60% of the pupae treated with 1 ng of ds*TcCda1* were able to eclose, but the resulting adults exhibited impaired leg movement (indicated by arrows; see Movie S1). (*B*) Legs dissected from the dsRNA-treated day 5 pupae were incubated with 10 M NaOH, followed by staining for chitin with FITC-CBD (green). The internal tendon cuticle junction (or breaks) between femur and tibial leg segments is indicated by white (or yellow) arrows. (*C*) Cryosections of the legs from dsRNA-treated day 5 pupae were incubated with FITC-CBD, Alexa Fluor 546-phalloidin, and TO-PRO3 to stain chitin (green), actin (red), and nuclei (blue), respectively. A representative region of muscle fiber (MF) attachment to ITC is enlarged in the *Insets*. AC, adult cuticle; CUT, internal tendon cuticle. (*D*) Morphology of the femoral muscles of indicated dsRNA-treated day 5 pupae analyzed by TEM. Arrowheads indicate Z-discs of muscles.

basal matrix and the other end adhered to the exoskeletal tendon cell (cuticular chitin stained green) (Fig. 1 *C*, *Left*). In contrast, most of the femur muscles in adult insects depleted of TcCDA1, TcCDA2a, or TcCDA1/2a were detached from both the internal and exoskeletal tendon cells, indicating an absence of attachment at either end (Fig. 1 *C*, *Center* three panels). Examination of control muscle ultrastructure by TEM revealed an organized arrangement of parallel myofibers characterized by regularly spaced Z-discs (arrowheads in Fig. 1 *D*, *Left*). The TcCDA1-depleted insects had rounded muscles and a complete loss of sarcomere patterning (Fig. 1*D*, second panel from the left). Depletion of TcCDA2a or TcCDA1/2a led to myofibers with unorganized sarcomeres and a streaming Z-disk morphology (Fig. 1*D*, third and fourth panel from the left). TcCDA2b depletion did not significantly alter muscle morphology as compared to the ds*TcVer*-treated control tissue (Fig. 1 *D*, *Right*), indicated by the relatively normal muscle structure revealed by phalloidin staining and TEM images.

CDA Deficiency Does Not Affect Early Muscle Development and Attachment. To obtain detailed information on the timing of the internal tendon cuticle breakage and muscle detachment following RNAi for *TcCda*s, confocal microscopic analyses of

sections of developing femur–tibia junctions were conducted at three time points, pupal day 4 (P4), early pupal day 5 (EP5), and late pupal day 5 (LP5). After staining for muscles (red) and cuticular chitin (green), the ds*TcCda*-treated femur–tibia sections were compared to ds*TcVer*-controls (Fig. 2 *A*–*C*). On P4 (Fig. 2*A*), the internal tendon cuticle connecting the femur and tibia was continuous and there was no evidence of tendon breakage following RNAi for either *TcCda1*, *TcCda2a*, or *TcCda2b* (arrows pointing to cuticular chitin [green] marked with the letters "CUT" in Fig. 2 *D*, *G,* and *J*). The myofibers had developed normally, exhibited the same stretched morphology and sarcomere patterning as the ds*TcVer*-control femurs, and appeared to be connected at one end to the ITC and to the ETC at the other end. Early on day 5 (Fig. 2, *Center* panels labeled EP5), there was evidence of tendon cuticle breaks (indicated by the boxed region) and rounding of muscles in TcCDA1-depleted femurs (Fig. 2*E*), but not in the TcCDA2a-depleted samples (Fig. 2*H*). Near the end of day 5 of the pupal period (Fig. 2, *Right* panels labeled LP5), the internal tendon cuticles were clearly broken, the femur cuticle (stained green for chitin) was retracted from the tibial joint in both TcCDA1- and TcCDA2a-depleted samples, and the muscles were rounded (Fig. 2 *F* and *I*). Myofibers in ds*TcCda2b*-treated samples remained stretched and displayed normal sarcomere patterning similar to controls at all stages tested (Fig. 2 *J*–*L*).

On EP5, the internal tendon cuticle connecting the femur and tibia was continuous in ds*TcVer*-control and TcCDA2a-depleted samples (*SI Appendix*, Fig. S4). The majority (~85%) of the femur–tibia cuticle joints from TcCDA1-depleted insects were ruptured on EP5, while the remaining 15% of the insects had intact cuticle joints (*SI Appendix*, Fig. S4). There was no evidence of tendon breakage in TcCDA2a- or TcCDA2b-depleted insects at EP5. Later at the LP5 stage, the internal tendon cuticle had ruptured near the femur–tibia joint, and the chitinous laminae were partially unraveled in all TcCDA1- and TcCDA2a-depleted (Fig. 3 *D* and *F*) but not in ds*TcVer*-control or TcCDA2b-depleted insects (Fig. 3 *C* and *E*), suggesting that this joint had become mechanically vulnerable due to TcCDA1 or TcCDA2a deficiency. Fig. 3 *A* and *B* represent cartoon representations of the summary of all the findings and focuses on the connections or the lack thereof between ETCs and ITCs (with their attached cuticles) that bridge the myofibers as seen on P5. The dorsal internal tendon of the femur linking more muscle fibers than the ventral one appears to also form a thicker cuticle, particularly in its proximal region, presumably in order to balance out the greater tension subjected to the former. We propose that the development and attachment of muscles to the tendon cells are unaffected by a deficiency of TcCDA1 or TcCDA2a proteins until P4. However, when there is a deficiency of either CDA protein, this attachment is not strong enough to withstand persistent strong muscle contractions and limb movements associated with the onset of adult eclosion that occur late on P5, leading to rupture and unraveling of both the dorsal and ventral internal tendon cuticles.

Tendon Cells at Both Ends of Muscle Mature to Provide Mechanical Links to Exoskeletal and Internal Tendon Cuticles. To understand the role of tendon cells as transducers of muscle contractive forces to the cuticle during pupal MTJ development, detailed TEM analyses were performed using sections of *T. castaneum* femur tissue from ds*TcVer*-control insects at multiple time points (P4, EP5, and LP5), paying particular attention to the following joints: exoskeletal cuticle–ETC, the two MASs (ETC-myotube and myotube-ITC), and ITC-internal tendon cuticle (Fig. 4). On P4, the ETCs were connected at their basal side to the myotubes, presumably involving integrins embedded in the membranes of either cell type (9). The plasma membrane of the myotube was flat and parallel to the tendon cell basal membrane (arrow marked MTJ in Fig. 4*A*) with no evidence of interdigitation into the tendon cell basal membrane (or basement membrane). A small space separates the two cell types, which may represent the ECM as described by Prokop et al. (9) (Fig. 4 *A* and *J*). The ETCs were spherical/oval and compact with no discernable microtubules. However, on EP5, the ETCs appeared elongated and the microtubules within tendon cells became such prominent structural elements by the LP5 stage that other cellular details became obscured (Fig. 4 *F* and *I*; *SI Appendix*, Fig. S6 for confirmation that these are microtubules). These stretched and parallel microtubules are of the same orientation as the myofibers in the muscle cells below them and connected with the apical electron-dense HAJs just below the cuticle. The HAJs became more numerous at LP5, suggesting growth of microtubules toward the cuticle (Fig. 4 *C* and *F*). Additional long fibrous extensions originating in the HAJs and oriented in the same direction as microtubules appear to extend into the cuticular laminae at this stage (yellow arrows in Fig. 4*F*; see also Fig. 6 *E* and *H* for sharper images). These extensions might represent the "tonofilaments" described previously in insects and other arthropods, but they are not electron-dense (5, 9, 35, 36).

The basal ends of several apicobasally oriented parallel microtubules were attached to the electron-dense MTJs that connect the basal tendon cell membranes to myotubes (Fig. 4*L*). The microtubules traversed the entire length of the tendon cells (Fig. 4*I*) and appeared to pull in the basal plasma membranes, causing the closely apposed myotube membranes with attached myofibers to become interlocked with tendon cell membranes. These MTJs have the shape of a connected series of pointy, inverted V-shaped membrane interdigitations. The tendon cell microtubules were fully stretched in the apicobasal direction and were oriented in the same direction as the muscle fibers, suggesting that they represent linked components in force transmission to the exoskeleton.

This exoskeletal cuticle–ETC–muscle linkage is mirrored at the other end of the myotubes where they meet ITCs with their attached internal tendon cuticle (marked "CUT" in Fig. 5 *A* and *I*). The MTJs near the internal tendon cuticle also show enhanced membrane surface contacts and membrane interdigitations between the muscle and tendon cells (Fig. 5*E*). The ITCs also have extensive microtubules that run in the same direction as myofibers and connect the HAJs and MTJs in ITCs. Unlike the ETCs, the fibrous extensions from the HAJs of ITCs appear to interact more extensively with the cuticular laminae and to display a beaded structure (Fig. 5*I*). Thus, the two chitin-containing cuticles with their attached tendon cells (i.e., ETCs and ITCs) made semirigid by microtubules at the two ends of myotubes provide Velcro-like joints to the two ends of the spring-like myotubes, permitting the transmission of force from muscle contraction to the exoskeleton and thus enabling movement.

CDA Deficiency Results in Alterations in Morphology of Tendon Cells and MTJs. In addition to internal tendon cuticle breaks, structural changes were observed in tendon cells and MTJs after down-regulation of *TcCda* transcripts. TEM analyses of the force transmission apparatus, consisting of the exoskeletal cuticle–ETC–myotubes–ITC–internal tendon cuticle in femur sections of day 5 pupae, are shown in Figs. 5 and 6. The tendon cells of control femurs had parallel microtubules that connected the electron-dense HAJs and MTJs, found at either the

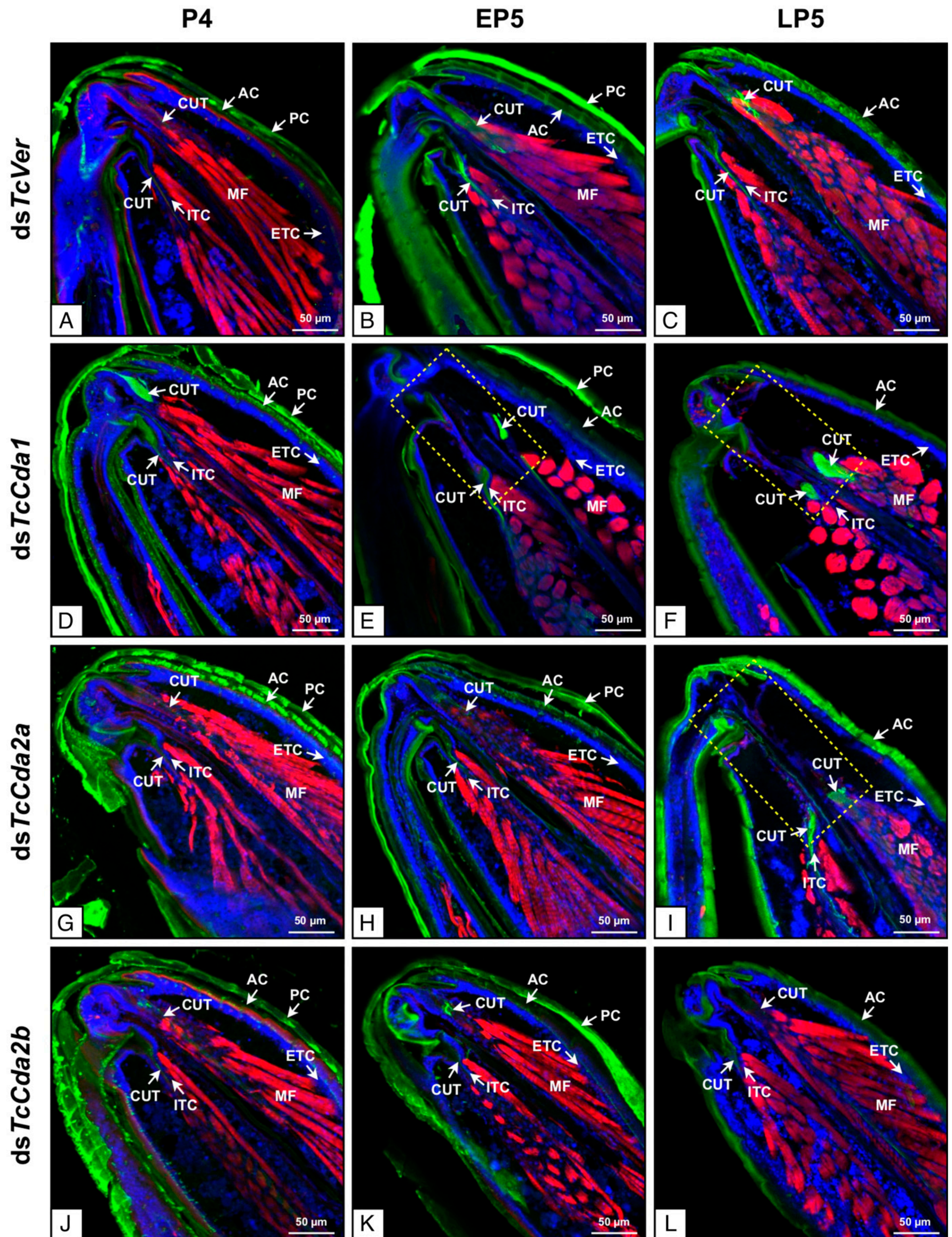


Fig. 2. Timing of internal tendon cuticle breakage in TcCDA1- and TcCDA2a-deficient insects. Cryosections of legs dissected from P4, EP5, and LP5 pupae that had been injected with ds*TcVer* (*A*–*C*), ds*TcCda1* (*D*–*F*), ds*TcCda2a* (*G*–*I*), or ds*TcCda2b* (*J*–*L*) on day 0 pupal stage were incubated with FITC-CBD, Alexa Fluor 546-phalloidin, and TO-PRO3 to stain chitin (green), actin (red), and nuclei (blue), respectively. Yellow dotted line box indicates the breakage point in internal tendon cuticle. MF, muscle fiber; PC, pupal cuticle.

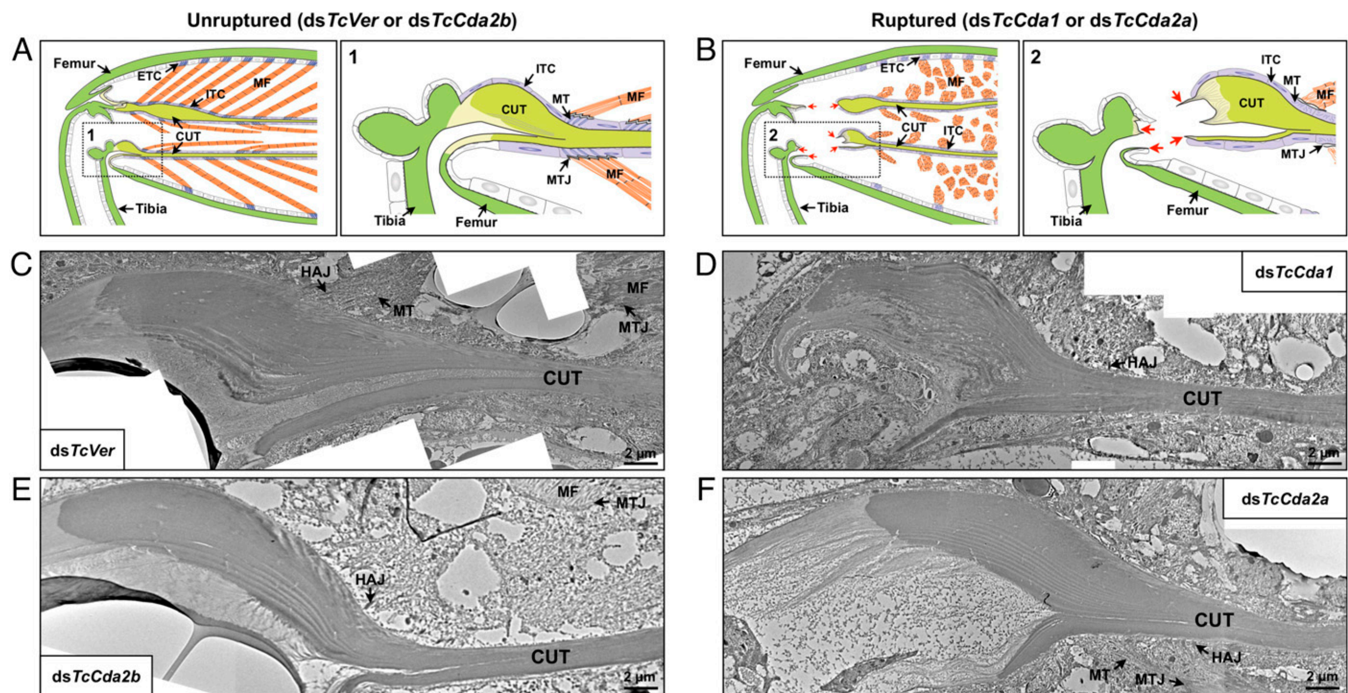


Fig. 3. Morphology and break-point of internal tendon cuticles of femurs after RNAi. Schematic diagram of femur segments illustrating the unruptured (*A*) and ruptured (*B*) internal tendon cuticles of femurs and detachment of femur muscles from ETCs and ITCs on P5 following RNAi for *TcVer* (*C*) or *TcCda2b* (*E*) and *TcCda1* (*D*) or *TcCda2a* (*F*), as indicated. One of the internal tendon cuticle junctions between the tibial and femur segments (dotted line boxes 1 and 2 in *A* and *B*) is enlarged, *Right*. The internal tendon cuticle breaks between femur and tibial leg segments are indicated by red arrows in *B*. (*C*–*F*) Ultrathin sections of the legs from insects at LP5 stage treated with ds*TcVer*, ds*TcCda1*, ds*TcCda2a*, or ds*TcCda2b* (on P0) were prepared for ultrastructure analysis of the internal tendon cuticles by TEM. MT, microtubule. The following color coding is used: green, chitin (exoskeletal cuticle and internal tendon cuticle); orange, muscle; purple, ETC and ITC; blue lines inside cells indicate microtubules.

apical or basal ends of the tendon cells, presumably to help maintain/transmit force along the posterior/anterior axis of the tendon cells and to keep those cells elongated (Fig. 6*A*). Administration of dsRNA for *TcCda1* or *TcCda2a*, but not *TcCda2b*, resulted in distinct changes in subcellular architecture of the tendon cells. On P5, both microtubules and HAJs were missing or greatly diminished in the TcCDA1- and TcCDA2a-depleted insect femurs (compare Fig. 6 *F* and *G* with *E* and *H*). The MTJs of CDA-depleted femurs lacked the characteristic zigzag membrane interdigitations (compare Fig. 6 *J* and *K* with *I* and *L*). Instead, there were gaps between tendon cells and muscle cells (red arrowheads in Fig. 6 *B* and *C*), consistent with the muscle detachment phenotype observed in confocal analyses (Fig. 2). Where MTJs could be discerned, they were flat, resulting in greatly decreased contact surface areas at the boundaries of the tendon cells and muscle cells (best illustrated in Fig. 6 *J* and *K*). Also notable was the absence of intracuticular fibers (yellow arrows) in TcCDA1- and TcCDA2a-deficient femurs (compare Fig. 6 *F* and *G* with *E* and *H*). This aspect will be discussed later.

Similar phenotypes were also observed at the other end of the myotubes where they meet ITCs with their attached internal tendon cuticle (Fig. 5). The extensive membrane interdigitations between myotubes and ITCs present in control femurs (marked MTJ in Fig. 5 *A* and *E*) were replaced by flat and discontinuous connections with a greatly reduced contact surface area between the two cell types (compare Fig. 5 *F* and *G* with *E* and *H*). Furthermore, the extensive microtubules connecting the apical and basal HAJs and MTJs of ITCs were either absent or deficient in TcCDA1- and TcCDA2a-depleted femurs. The myofibers were noticeably less stretched in these two treatments compared to the control (compare Fig. 5 *F* and *G* with *E*) and became rounded once they were completely detached from the ITCs (*SI Appendix*, Fig. S5). Together, these data suggest that deficiency of TcCDA1 or TcCDA2a not only compromises muscle structure but also alters the MTJ architecture and stability by reducing the overall contact surface area between the membrane systems of these two cell types, and also by interfering with tendon cell microtubule organization with the loss of HAJs. These alterations are visible in MTJs involving both ETCs and ITCs.

CDA Deficiency Leads to Disruption of Microtubules. To investigate how TcCDA deficiency affects microtubule organization within tendon cells, we used an α-tubulin antibody to label TEM sections of femur tissues at the LP5 stage (*SI Appendix*, Fig. S6). In ds*TcVer*-injected controls as well as in femurs from ds*TcCda2b*-treated animals, tubulin labeling indicated that parallel bundles of tendon cell microtubules extending from the MTJs terminated in HAJs and stopped short of the ETC-attached cuticle (*SI Appendix*, Fig. S6, *Left* and *Right*). These data suggest that while microtubules contributed to maintenance of the HAJs and shape of tendon cells, they are not directly involved in cuticle attachment. However, other fibers of lower electron density extended from the HAJs and penetrated several layers of the chitin laminae in the cuticle (yellow arrows in *SI Appendix*, Fig. S6*A*; see also Fig. 6 *E* and *H*), suggesting that they may contribute to the stabilization of the cuticle-tendon cell junctions. In TcCDA1- or TcCDA2a-depleted femurs, the microtubule arrays and HAJs were less organized, (compare *SI Appendix*, Fig. S6 *F* and *G* with *E* and *H*) and not particularly abundant near MTJs or just below the interface with the exoskeletal cuticle. The relatively flat MTJs lacked the extensive membrane interdigitation, presumably due to a deficiency/disorientation of MTJ/HAJ-associated microtubules (*SI Appendix*, Fig. S6 *J* and *K*). In contrast, the control

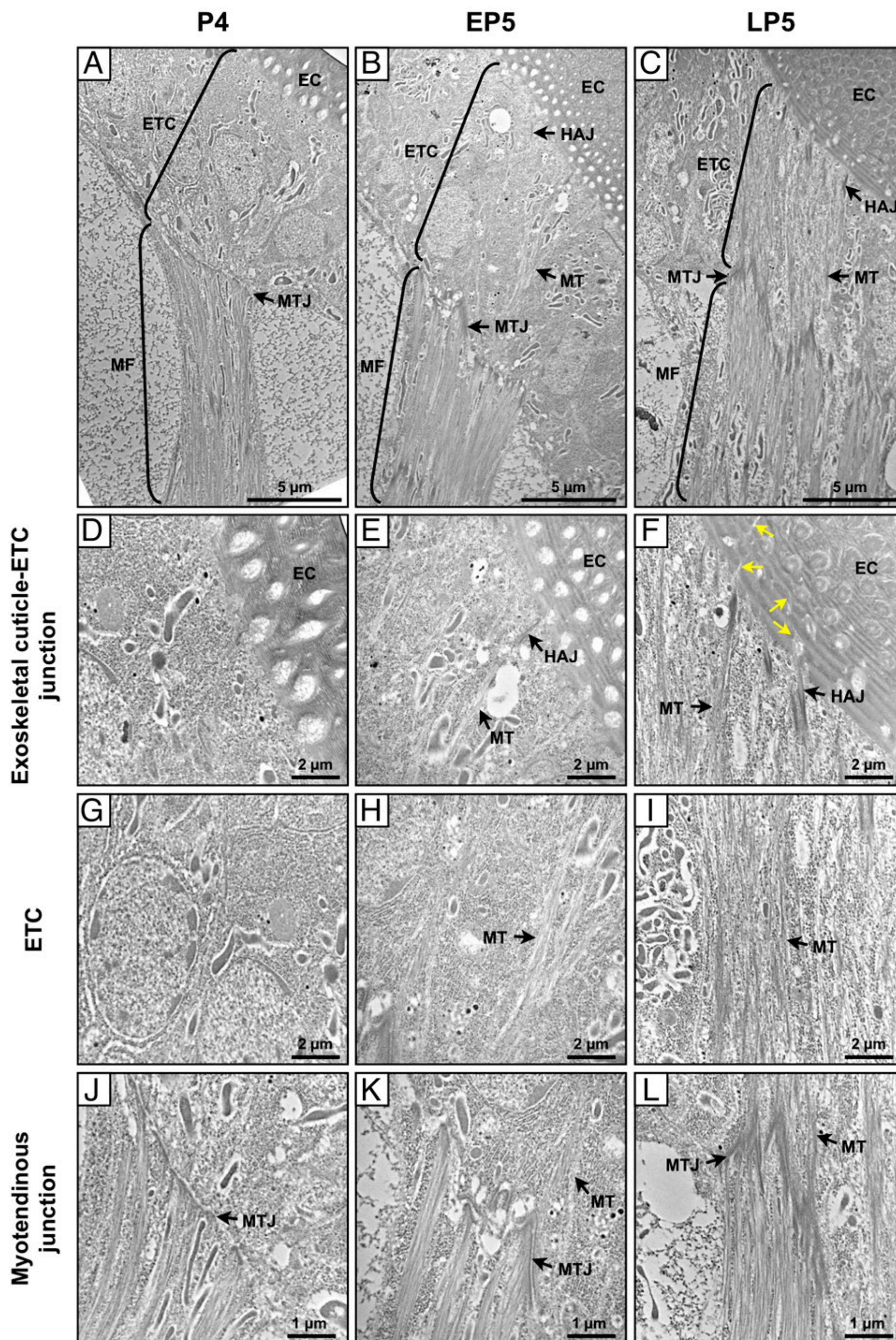


Fig. 4. Maturation of microtubule arrays and adherens junctions during pupal development. (*A*–*C*) Legs were dissected from P4, EP5, and LP5 ds*TcVer*-treated (control) pupae for analysis of ultrastructure by TEM. Larger magnifications of the exoskeletal cuticle–ETC junction (*D*–*F*), the internal ETC (*G*–*I*), and the MTJ between ETC and muscle fiber (*J*–*L*). EC, exoskeletal cuticle. Yellow arrows indicate translucent intracuticular fibers.

and TcCDA2b-deficient insects had numerous MTJ/HAJs with attached parallel bundles of microtubules, which appeared to pull the myotubule membranes into the tendon cell body (*SI Appendix*, Fig. S6 *E*, *H*, *I*, and *L*).

Depletion of the ZPD Protein TcDumpy Leads to Loss of Intracuticular Fibers. Several ZPD-containing proteins expressed in specialized epidermal cells in *D. melanogaster* embryos and wing cells as well as at limb MASs attach the apical plasma membrane to the adjacent cuticle. They also have been suggested to play a role in both cell–cell adhesion and cytoskeletal reorganization (5, 17, 18). To investigate whether ZPD proteins contribute to the formation of intracuticular fibers in tendon cells, we performed RNAi of the *T. castaneum* ortholog of the well-characterized *D. melanogaster* ZPD protein, Dumpy (18, 37). Down-regulation of transcripts for *Tcdumpy* resulted in muscles that were not fully attached to the basal matrix of tendon cells especially at the edges, suggestive of a reduced area of contact between the two cell types, loss of motility, and lethality at the pharate adult stage (compare Fig. 7 *A* with *B*). TEM analyses of femur sections following RNAi of *Tcdumpy* did not result in gross alteration of laminar organization or pore canals in the cuticle (compare Fig. 7 *A* with *B*). However, the electron-lucent intracuticular fibers visible in the endocuticular layers of controls were not present in multiple sections from the TcDumpy-deficient insects (compare Fig. 7 *E* with *F*), similar to results obtained from RNAi of *TcCda1* and *TcCda2a* (Fig. 6 *F* and *G*). Surprisingly, the apical membrane of the tendon cells (both ETCs and ITCs) remained flat and attached to the endocuticle (Fig. 7*F*), but the attachment to the myofibers was altered,

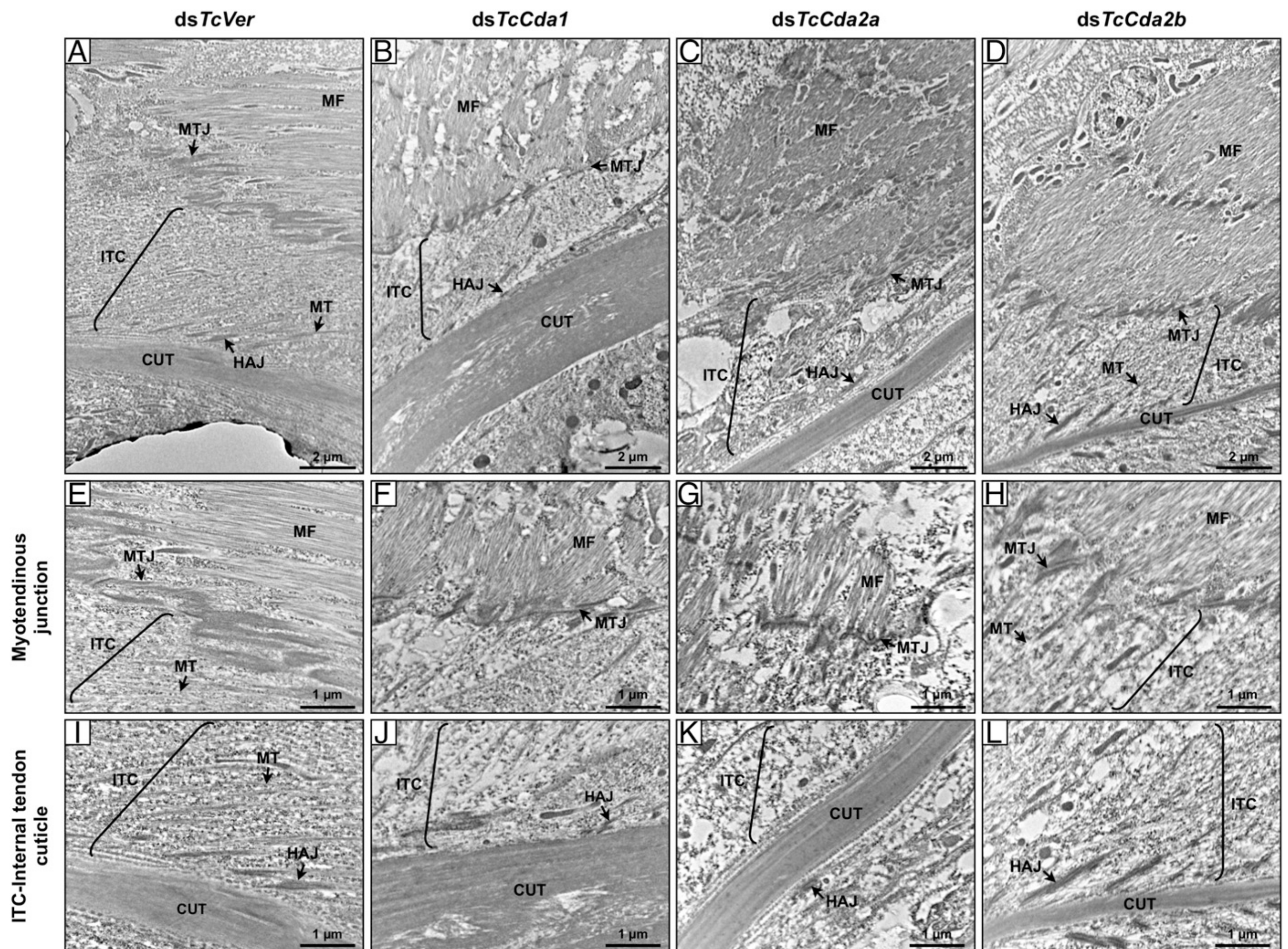


Fig. 5. Ultrastructure of the internal tendon cuticles, ITCs, and muscle fibers of femurs from control and TcCDA-depleted insects. (*A*–*D*) Legs were dissected from day 5 pupae treated (on P0) with ds*TcVer*, ds*TcCda1*, ds*TcCda2a*, or ds*TcCda2b* for analysis of ultrastructure by TEM. Larger magnifications of the MTJ between muscle fiber and ITC (*E*–*H*) and the ITC-internal tendon cuticle junction (*I*–*L*).

denoted by flat MTJs and greatly reduced membrane contacts between the tendon cell and myotubes (Fig. 7*J* and *SI Appendix*, Fig. S7 *B* and *F*). Also notable was the fact that the microtubule network was not apparent in both ETCs and ITCs (Fig. 7*B* and *SI Appendix*, Fig. S7*B*). However, apical plasma membrane protrusions (APMPs) that penetrate the cuticles and pore canals lying just above APMPs were not affected after RNAi for *Tcdumpy* (Fig. 7 *B* and *F*). These results indicate that Dumpy is involved in maintenance of tendon cell architecture, but not in gross cuticle integrity.

Do CDAs and Dumpy Participate in the Same Force Transmission Chain? The results presented so far show that RNAi of *TcCda1*, *TcCda2a*, and *Tcdumpy* produced similar phenotypes in tendon cells and myofibers of *T. castaneum* femur segments even though their effects on the cuticle architecture were different. To investigate how CDAs and Dumpy proteins interact in force transmission, we performed RNAi using combinations of dsRNAs for *TcCda1* and *Tcdumpy* (ds*TcCda1/dumpy*) or *TcCda2a* and *Tcdumpy* (ds*TcCda2a/dumpy*) (Fig. 7). The phenotypic effects of these double RNAi treatments on ETCs and muscles were similar to those seen with either *TcCda1*, *TcCda2a*, or *Tcdumpy* RNAi alone, including loss of apical HAJs, flat MTJs with reduced membrane interdigitation, loss of organized microtubules in tendon cells, rounded muscles, and loss of intracuticular fibers (Fig. 7 *C*, *D*, *G*, *H*, *K*, and *L*). The ITCs and the attached myofibers also displayed a similar loss of HAJs, flat MTJs, and loss of myofiber stretching, mirroring the phenotype seen with ETCs and the MTJs (*SI Appendix*, Fig. S7 *C*, *D*, *G*, *H*, *K*, and *L*). However, there was a noticeable difference in the integrity of the internal tendon cuticle joint between the femur and tibial segments. This joint remained unbroken in insects treated with ds*Tcdumpy* alone compared to the TcCDA1- or TcCDA2a-depleted insects that did exhibit a ruptured internal tendon cuticle (*SI Appendix*, Fig. S8; compare with Fig. 3). Interestingly, even in femurs from insects treated with ds*TcCda1* + ds*Tcdumpy* (ds*TcCda1/dumpy*) or ds*TcCda2a* + ds*Tcdumpy* (ds*TcCda2a/dumpy*), this joint remained unbroken as revealed by FITC-CBD staining and TEM analysis, likely because no muscle contractive force was conveyed to this vulnerable joint in the absence of TcDumpy (*SI Appendix*, Figs. S8 *B*–*D*, *G*, and *H*, and S9).

Discussion

Myogenesis in vertebrates and the model dipteran insect species, *D. melanogaster*, is influenced by tendon and ECM development, and largely follows similar pathways (1, 2). Relatively few studies have focused on the relationship between insect exoskeletal cuticle development in concert with limb muscle and internal tendon cuticle

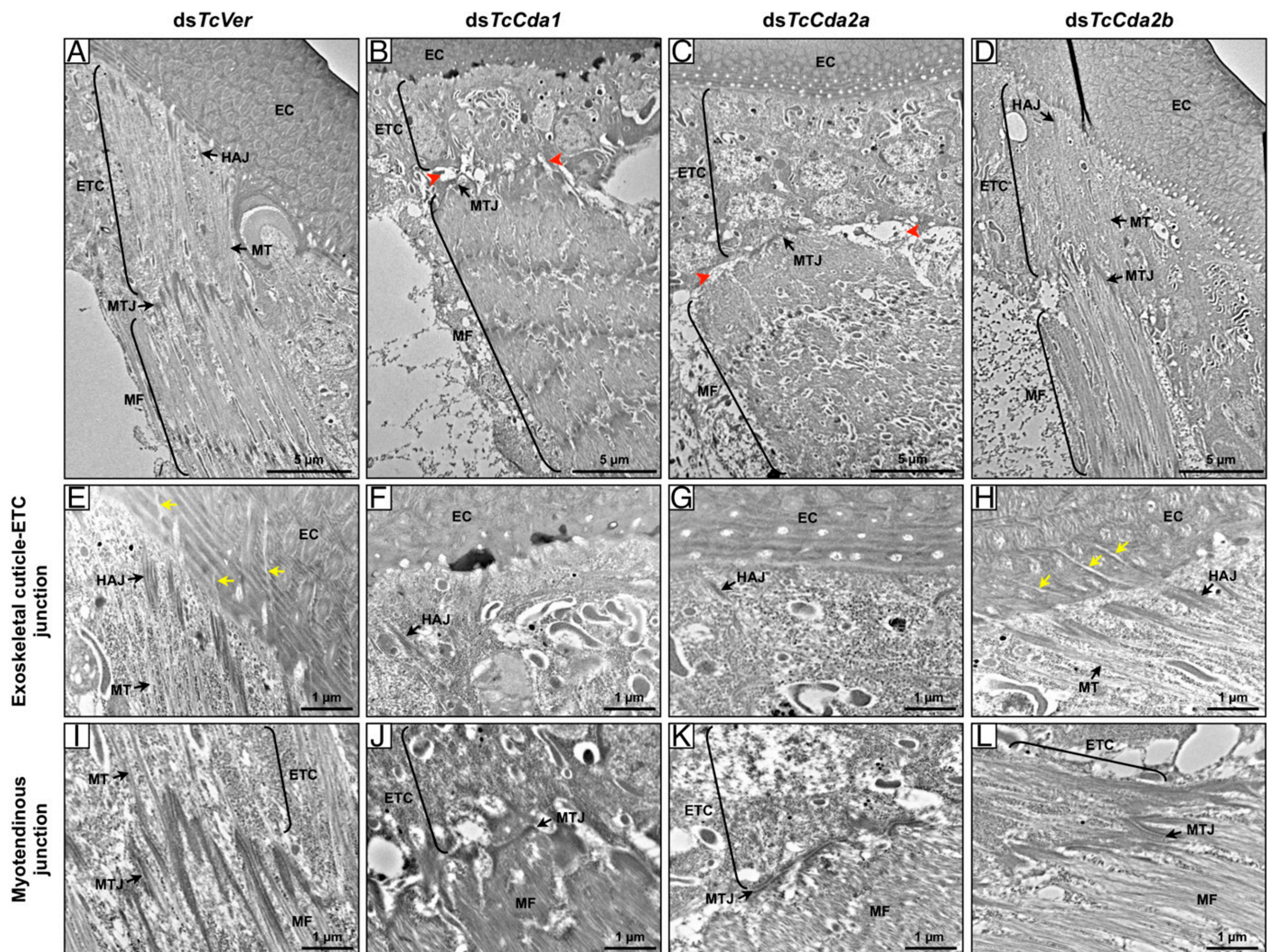


Fig. 6. Morphology of femoral exoskeletal cuticle–ETC–muscle junctions after RNAi. (*A*–*D*) Ultrathin sections of femurs from day 5 pupae treated with ds*TcVer*, ds*TcCda1*, ds*TcCda2a*, or ds*TcCda2b* were prepared to analyze the ultrastructure of the exoskeletal cuticle–ETC–muscle junctions. Larger magnifications of the exoskeletal cuticle–ETC junction (*E*–*H*) and the MTJ between ETC and muscle fiber (*I*–*L*). Red arrowheads in *B* and *C* indicate partial muscle detachment from the ETCs. Yellow arrows in *E* and *H* indicate intracuticular fibers.

development, microstructure, and locomotion (9, 18, 33–36). Besides the transmembrane integrins, several integrin-binding proteins—such as tiggrin, thrombospondin, and laminins—are involved in cell–cell and cell–matrix adhesion (38–41). *D. melanogaster* pupal lethal *fon* mutant larvae that are deficient in this secreted ECM protein also have detached muscles and decreased larval motility (41). The phenotypes reported in this study, obtained from the model coleopteran species, *T. castaneum*, following depletion of two secreted enzymes—TcCDA1 and TcCDA2a that are presumed to participate in the deacetylation of chitin in the cuticular matrix to form a chitin-chitosan heteropolysaccharide—are similar to those reported in the model dipteran, *D. melanogaster*, upon loss of integrin or other proteins involved in adhesion, indicating an essential and independent role for CDAs in adhesion/myogenesis, which has not been recognized previously.

The maturation of insect muscles is regulated by force perception during myogenesis, which occurs in association with the development of both ITCs and ETCs (10, 33). Contraction followed by expansion of myofibers and the build-up of tension is essential for muscle development and maturation (2, 10, 16, 42, 43). In *D. melanogaster*, genetic interference with myotube attachment to tendon cells or laser-mediated ablation of tendon cell extensions, leads to defects in myofibrillogenesis (16).

CDAs Are Important for Maintaining Internal Tendon Cuticle Integrity and Tendon Cell Morphology. Up to now there have been no reports about the influence of chitin/chitosan or chitin-modifying enzymes on the maturation and attachment of muscles to the ECM or in locomotion of insects. The time-course studies reported here indicate that the requirement for CDA in the development of MTJs is not manifested in the early stages of leg muscle development because the attachment of myotubes to ITCs and their elongation toward MASs in the epidermis are unaffected by TcCDA depletion, at least until EP5 (Fig. 2). Even the attachment of the apical plasma membrane of either ITCs or ETCs to the chitinous endocuticle appears unchanged by a deficiency of TcCDA1 or TcCDA2a (Figs. 5 and 6). Strikingly, however, TcCDAs are essential for maintenance of the functional integrity of the internal leg tendon cuticle and muscles around the time insects display strong muscle contractions and leg movements prior to adult eclosion on P5. The pulling force of muscle contraction likely leads to a snapping of the internal tendon cuticle connecting the femur and tibia, and the subsequent rounding of muscles, due to a weakening or loss of elasticity of the internal tendon cuticle, resulting from failure to deacetylate the chitin matrix. The reason why the tendon cuticle snaps at this location is not clear. We could not correlate this cuticular weak spot with the

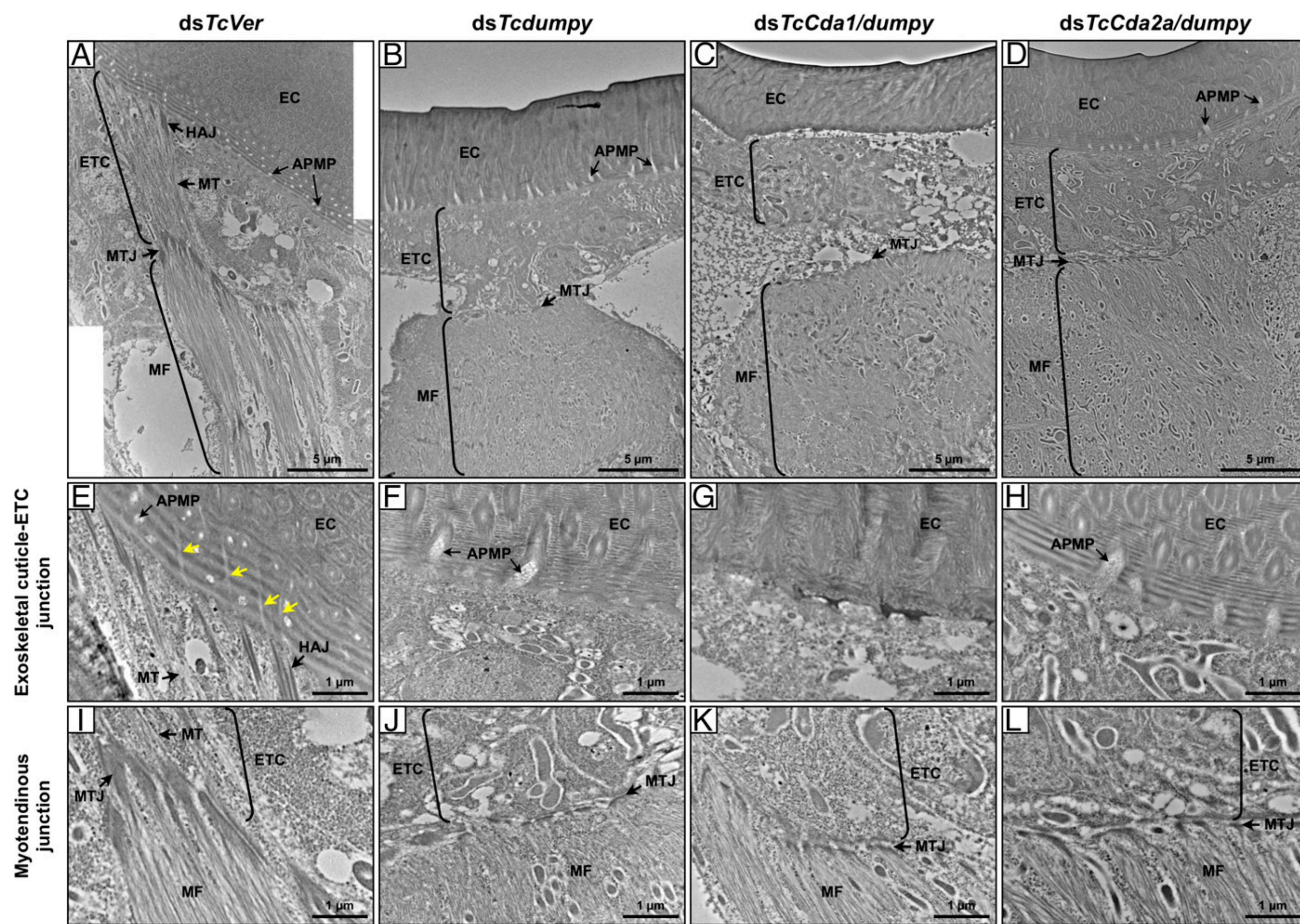


Fig. 7. Ultrastructure of femoral exoskeletal cuticle–ETC–muscle junctions in TcDumpy-, TcCDA1/Dumpy-, and TcCDA2a/Dumpy-depleted insects. (*A*–*D*) Ultrathin sections of femurs from day 5 pupae treated with ds*Tcdumpy*, ds*TcCda1/dumpy*, ds*TcCda2a/dumpy*, or ds*TcVer* were prepared to analyze ultrastructure of the exoskeletal cuticle–ETC–muscle junctions. Larger magnifications of the exoskeletal cuticle–ETC junction (*E*–*H*) and the MTJ between ETC and muscle fiber (*I*–*L*). Yellow arrows in *E* indicate intracuticular fibers.

expression level of CDA by tendon cells near these joints. We also could not attribute the weakness of the femur–tibia cuticle joint to the differences in the thickness of the dorsal and ventral internal tendon cuticle near this joint (Fig. 3) because both the dorsal and ventral fork are equally susceptible to snapping and fraying of chitinous fibers. We propose that this cuticular joint experiences the most bending stress at the time of strong leg movements, which occur on P5. This interpretation is further supported by the finding that when force distribution is interrupted by down-regulation of TcDumpy, this joint is not broken at the time of muscle contraction, even when TcCDA1 or TcCDA2a are depleted.

The earliest observable phenotype of TcCDA depletion appears to be the failure to properly assemble bundles of microtubules on P5. The microtubules fail to connect the HAJs just below the cuticle to the MTJs at the base of tendon cells, and the myotubes attached to tendon cells exhibit loss of shape and structural integrity. The absence of microtubule networks and MTJs at the basal side of tendon cells leads to a loss of V-shaped membrane interdigitations that are normally needed to substantially increase the surface area of contact between tendon and muscle cells to strengthen adhesion of both ETCs and ITCs to myotubes. Attachment of the tendon cell apical membrane to the endocuticle appears to be facilitated by additional fiber-like structures of unknown identity, which extend from apical HAJs and penetrate a few of the endocuticular laminae. These intracuticular fibrous connections between the tendon cells and the cuticular laminae also fail to develop in TcCDA-deficient tendon cells. These observations indicate that CDA deficiency affects not only cuticle organization but also the structural properties of the underlying tendon cells.

Microtubule Integrity and ZPD Proteins Are Essential for Maintaining Muscle Tension. How does a deficiency of CDAs that are secreted and primarily located at the base of the endocuticle affect the morphology and function of muscles that are not directly attached to the cuticle? This is not merely due to a weakened or abnormal cuticle due to defective deacetylation of chitinous laminae. First, we observe cuticle snapping even when the cuticular laminae in both external and internal tendon cuticles appear normal, as in the case of depletion of TcCDA2a (in contrast to TcCDA1 depletion, which does lead to cuticular abnormalities). Furthermore, even when there are obvious gross cuticular and wing abnormalities when we deplete some critical structural cuticular proteins, we do not see defective locomotion (44, 45). One example is shown in *SI Appendix*, Fig. S10. In this case, transcripts for a specific chitinase involved in chitin maturation, *TcCht7*, were depleted by RNAi. In these pharate adults, the external and internal tendon cuticles are abnormal, but the insects have a normal gait and there is no snapping of the femur–tibia internal cuticle joint. We propose that CDAs indirectly modify the properties of the cytoskeleton of underlying tendon cells by influencing the

development of microtubules and HAJs in these cells. The tendon cell is loaded with microtubule arrays that are oriented in the same direction as myofibers during *T. castaneum* P5. This time period coincides with tendon cell elongation and morphological changes occurring within developing muscles that are also being subjected to increased resistive tension. Arrays of F-actin fibers overlapping with specialized microtubules with 15 tubulin protofilaments and myosin II have been reported in the cytosol of wing cells and in tendon cells of *D. melanogaster* (12, 46, 47). It has been proposed that these microtubule arrays play a critical role in stiffening tendon cells presumably assisted by cross-linking or stabilization of microtubules mediated by proteins, such as Shortstop/Spectraplakin and the microtubule minus-end binding protein, Patronin (48, 49).

Further stabilization of the adhesion of tendon cell apical plasma membrane to the cuticle may be provided by fibers emanating from HAJs, which appear to penetrate the laminar cuticle in external tendons of control insects during P5; these fibers are sparse or absent in tendon cells of ds*TcCda1*- and ds*TcCda2a*-treated insects. Previous work suggested that the link between the tendon cell and cuticle involves electron-dense fibers variously denoted as tonofilaments, muscle attachment fibers, or intracuticular fibers that emanate from (or are embedded in) plasma membrane pits (5, 36, 50, 51). The chemical nature of these fibers has not yet been determined (18). These fibrous extensions may be transmembranous proteins that have a cytosolic tail anchoring them on HAJs, crossing the apical plasma membrane and penetrating several laminae of the procuticle to terminate in the endocuticular layers.

We hypothesize that TcCDA-deficiency leads to a failure to develop or anchor these filaments in the endocuticle and that this process is essential for HAJ development in tendon cells at MAS. The tendon cell microtubules appear to cluster around and terminate in these HAJs, presumably stabilizing them. We have focused on one of the ZPD proteins, Dumpy, because it spans the apical membrane, and has a cytosolic tail capable of interacting with cytoskeletal elements, as well as extracellular domains that can interact with a variety of ligands. Dumpy and other ZPD proteins, such as Piopio and Papillote, are implicated in adhesion to the cuticle, while others play a role in microtubule stabilization (5, 17, 18). Relevant to this study, in *D. melanogaster* these proteins have been reported to be required for connectivity between the cuticle and muscles (5, 17, 52). Dumpy also has been shown to be involved in the formation of force-resistant stretchable filaments in the ecdysial space at MASs in *D. melanogaster* (18).

CDAs and ZPD Proteins May Operate in a Common Pathway. Here we have shown that the phenotypes resulting from RNAi of *Tcdumpy* in *T. castaneum* are similar to that of *TcCda1* or *TcCda2a*. Similarities include loss of intracuticular fibers, altered tendon cell integrity, reduced muscle tension, and defective motility, suggesting that CDAs and ZPD proteins share a common pathway in affecting force transmission. However, these proteins do not compensate for each other because depletion of any one of these proteins alone results in similar tendon cell and muscle phenotypes. In addition, depletion of the TcCDA1 has phenotypic effects on external and internal tendon cuticles that are not exhibited by TcDumpy deficiency. In fact, depletion of TcDumpy can counter the snapping of the internal tendon femur–tibia cuticle joint, presumably by blocking force transmission of contracting muscles to vulnerable cuticle junctions weakened by a lack of TcCDAs. Therefore, we propose that CDAs act upstream of ZPD proteins by providing a mechanism for anchoring ZPD-domain proteins to the cuticle. Nevertheless, force transmission requires the presence of TcDumpy because in its absence, there is no damage to the internal tendon cuticle even when it has been weakened (lost its elasticity) by a deficiency of TcCDA1 or TcCDA2a. Both classes of proteins (CDAs and ZPD proteins) act by influencing the cytoskeletal architecture of tendon cells that connect the cuticle to the underlying muscles, suggesting that they share a common pathway in force transmission. Since the laminar architecture of the cuticle and its pore canal organization are unaffected by TcDumpy deficiency (except for the loss of intracuticular fibers), the CDAs have an entirely separate role in higher order organization of the cuticle (25).

Because deacetylation of chitin leads to creation of free amino groups of glucosamine that are positively charged at physiological pH, we hypothesize that regions of deacetylated chitin with several contiguous positive charges serve as electrostatic binding/capture/attachment regions for these fibrous extensions, which may have negatively charged ends. It is worth noting that CDA1 and CDA2 have in their N-terminal region a copy of the LDLa receptor domain that contains several clusters of negatively charged amino acids, which may involve Ca^{2+}-dependent interactions with other matrix-associated proteins (53). The unique finding of this research is the involvement of CDA-like proteins in cuticle–tendon–muscle morphogenesis and interconnectivity as an additional essential component that affects both muscle and cuticle morphology and function. Whether the abnormal maturation of microtubules and the deficiency of HAJs is due directly to a lack of partially deacetylated chitin or due indirectly to an uncharacterized role of CDAs that involves association of the chitin-chitosan heteropolysaccharide with other apical matrix proteins, remains the subject of future studies. The link between CDA and ZPD proteins, including Dumpy, remains unclear at this point and may involve a direct interaction between CDA and ZPD-domain proteins. Alternatively, another cuticular protein may mediate this interaction. This will best be resolved by direct biochemical studies with purified CDA and ZPD proteins.

Materials and Methods

Details of all materials, reagents, and protocols used in this study, including *T. castaneum* rearing, cloning a *Tcdumpy* cDNA, RNAi, chitin staining, immunohistochemistry, confocal microscopy, and TEM are given in *SI Appendix, Materials and Methods*.

Data Availability. The *Tcdumpy* cDNA sequence has been deposited in GenBank (accession no. OL006342). All other study data are included in the main text and supporting information.

ACKNOWLEDGMENTS. We thank Drs. Michael Kanost and Hans Merzendorfer for critical reading of the manuscript; the Animal and Plant Quarantine Agency of Korea for maintenance of the red flour beetles; and Dr. Kwang Ho Lee (Center for Research Facilities at the Chonnam National University) for technical assistance with the transmission electron microscopy analyses. This work was supported by National Research Foundation of Korea (NRF) Grants funded by the Korean government (MSIT) NRF-2018R1A2B6005106 and NRF-2021R1A2C1006645 (to Y.A.) and Basic Science Research Program through the NRF funded by the Ministry of Education NRF-2020R1I1A3066074 (to M.Y.N.). E.R.G is supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases of the NIH under Award R01AR060788.

Author affiliations: [a]Department of Applied Biology, Chonnam National University, Gwangju 61186, South Korea; [b]Department of Forest Resources, AgriBio Institute of Climate Change Management, Chonnam National University, Gwangju 61186, South Korea; and [c]Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS 66506

1. A. Subramanian, T. F. Schilling, Tendon development and musculoskeletal assembly: Emerging roles for the extracellular matrix. *Development* **142**, 4191–4204 (2015).
2. M. Valdivia, F. Vega-Macaya, P. Olguín, Mechanical control of myotendinous junction formation and tendon differentiation during development. *Front. Cell Dev. Biol.* **5**, 26 (2017).
3. C. W. Woodworth, The leg tendons of insects. *Am. Nat.* **42**, 452–456 (1908).
4. J. F. Vincent, U. G. Wegst, Design and mechanical properties of insect cuticle. *Arthropod Struct. Dev.* **33**, 187–199 (2004).
5. C. Bökel, A. Prokop, N. H. Brown, Papillote and Piopio: *Drosophila* ZP-domain proteins required for cell adhesion to the apical extracellular matrix and microtubule organization. *J. Cell Sci.* **118**, 633–642 (2005).
6. U. Tepass, V. Hartenstein, The development of cellular junctions in the *Drosophila* embryo. *Dev. Biol.* **161**, 563–596 (1994).
7. B. Alberts *et al.*, *Molecular Biology of the Cell* (Garland Science, New York, ed. 3, 1994).
8. M. Wehrli, A. DiAntonio, I. M. Fearnley, R. J. Smith, M. Wilcox, Cloning and characterization of alpha PS1, a novel *Drosophila melanogaster* integrin. *Mech. Dev.* **43**, 21–36 (1993).
9. A. Prokop, M. D. Martín-Bermudo, M. Bate, N. H. Brown, Absence of PS integrins or laminin A affects extracellular adhesion, but not intracellular assembly, of hemiadherens and neuromuscular junctions in *Drosophila* embryos. *Dev. Biol.* **196**, 58–76 (1998).
10. R. Schweitzer, E. Zelzer, T. Volk, Connecting muscles to tendons: Tendons and musculoskeletal development in flies and vertebrates. *Development* **137**, 2807–2817 (2010).
11. T. A. Bunch *et al.*, Characterization of mutant alleles of *myospheroid*, the gene encoding the beta subunit of the *Drosophila* PS integrins. *Genetics* **132**, 519–528 (1992).
12. J. Alves-Silva *et al.*, Prominent actin fiber arrays in *Drosophila* tendon cells represent architectural elements different from stress fibers. *Mol. Biol. Cell* **19**, 4287–4297 (2008).
13. F. J. Fogerty *et al.*, Tiggrin, a novel *Drosophila* extracellular matrix protein that functions as a ligand for *Drosophila* alpha PS2 beta PS integrins. *Development* **120**, 1747–1758 (1994).
14. N. H. Brown *et al.*, Talin is essential for integrin function in *Drosophila. Dev. Cell* **3**, 569–579 (2002).
15. B. Klapholz, N. H. Brown, Talin—The master of integrin adhesions. *J. Cell Sci.* **130**, 2435–2446 (2017).
16. M. Weitkunat, A. Kaya-Çopur, S. W. Grill, F. Schnorrer, Tension and force-resistant attachment are essential for myofibrillogenesis in *Drosophila* flight muscle. *Curr. Biol.* **24**, 705–716 (2014).
17. F. Roch, C. R. Alonso, M. Akam, *Drosophila miniature* and *dusky* encode ZP proteins required for cytoskeletal reorganisation during wing morphogenesis. *J. Cell Sci.* **116**, 1199–1207 (2003).
18. W. C. Chu, S. Hayashi, Mechano-chemical enforcement of tendon apical ECM into nano-filaments during *Drosophila* flight muscle development. *Curr. Biol.* **31**, 1366–1378.e7 (2021).
19. R. Dixit *et al.*, Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of *Tribolium castaneum* and three other species of insects. *Insect Biochem. Mol. Biol.* **38**, 440–451 (2008).
20. G. Tetreau *et al.*, Overview of chitin metabolism enzymes in *Manduca sexta*: Identification, domain organization, phylogenetic analysis and gene expression. *Insect Biochem. Mol. Biol.* **62**, 114–126 (2015).
21. Y. Xi, P. L. Pan, Y. X. Ye, B. Yu, C. X. Zhang, Chitin deacetylase family genes in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Mol. Biol.* **23**, 695–705 (2014).
22. W. Guo, G. Li, Y. Pang, P. Wang, A novel chitin-binding protein identified from the peritrophic membrane of the cabbage looper, *Trichoplusia ni. Insect Biochem. Mol. Biol.* **35**, 1224–1234 (2005).
23. Y. Arakane *et al.*, Analysis of functions of the chitin deacetylase gene family in *Tribolium castaneum. Insect Biochem. Mol. Biol.* **39**, 355–365 (2009).
24. L. Grifoll-Romero, S. Pascual, H. Aragunde, X. Biarnés, A. Planas, Chitin deacetylases: Structures, specificities, and biotech applications. *Polymers (Basel)* **10**, 352 (2018).
25. M. Y. Noh, S. Muthukrishnan, K. J. Kramer, Y. Arakane, Group I chitin deacetylases are essential for higher order organization of chitin fibers in beetle cuticle. *J. Biol. Chem.* **293**, 6985–6995 (2018).
26. R. Yu *et al.*, Helicoidal organization of chitin in the cuticle of the migratory Locust requires the function of the chitin deacetylase2 enzyme (LmCDA2). *J. Biol. Chem.* **291**, 24352–24363 (2016).
27. M. Zhang *et al.*, The putative chitin deacetylases Serpentine and Vermiform have non-redundant functions during *Drosophila* wing development. *Insect Biochem. Mol. Biol.* **110**, 128–135 (2019).
28. S. Muthukrishnan, S. Mun, M. Y. Noh, E. R. Geisbrecht, Y. Arakane, Insect cuticular chitin contributes to form and function. *Curr. Pharm. Des.* **26**, 3530–3545 (2020).
29. D. Zhao *et al.*, RNA interference-mediated functional characterization of group I chitin deacetylases in *Holotrichia parallela* Motschulsky. *Pestic. Biochem. Physiol.* **173**, 104770 (2021).
30. T. Zhang *et al.*, Group I CDAs are responsible for a selective CHC-independent cuticular barrier in *Locusta migratoria. Pestic. Biochem. Physiol.* **175**, 104854 (2021).
31. Y. Li, L. Liu, J. Yang, Q. Yang, An overall look at insect chitin deacetylases: Promising molecular targets for developing green pesticides. *J. Pestic. Sci.* **46**, 43–52 (2021).
32. J. F. Campbell, C. G. Athanassiou, D. W. Hagstrum, K. Y. Zhu, *Tribolium castaneum*: A model insect for fundamental and applied research. *Annu. Rev. Entomol.* **67**, 347–365 (2022).
33. C. Soler, M. Daczewska, J. P. Da Ponte, B. Dastugue, K. Jagla, Coordinated development of muscles and tendons of the *Drosophila* leg. *Development* **131**, 6041–6051 (2004).
34. C. Soler, L. Laddada, K. Jagla, Coordinated development of muscles and tendon-like structures: Early interactions in the *Drosophila* leg. *Front. Physiol.* **7**, 22 (2016).
35. J. B. Tucker *et al.*, Positioning and capture of cell surface-associated microtubules in epithelial tendon cells that differentiate in primary embryonic *Drosophila* cell cultures. *Cell Motil. Cytoskeleton* **57**, 175–185 (2004).
36. N. Znidaršič, P. Mrak, M. Tušek-Žnidarič, J. Strus, Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans. *ZooKeys* **176**, 39–53 (2012).
37. M. B. Wilkin *et al.*, *Drosophila* dumpy is a gigantic extracellular protein required to maintain tension at epidermal-cuticle attachment sites. *Curr. Biol.* **10**, 559–567 (2000).
38. B. Chanana, R. Graf, T. Koledachkina, R. Pflanz, G. Vorbrüggen, AlphaPS2 integrin-mediated muscle attachment in *Drosophila* requires the ECM protein Thrombospondin. *Mech. Dev.* **124**, 463–475 (2007).
39. T. A. Bunch *et al.*, The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila. Development* **125**, 1679–1689 (1998).
40. A. Subramanian, B. Wayburn, T. Bunch, T. Volk, Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in *Drosophila. Development* **134**, 1269–1278 (2007).
41. N. Green *et al.*, A common suite of coagulation proteins function in *Drosophila* muscle attachment. *Genetics* **204**, 1075–1087 (2016).
42. S. B. Lemke, T. Weidemann, A. L. Cost, C. Grashoff, F. Schnorrer, A small proportion of Talin molecules transmit forces at developing muscle attachments in vivo. *PLoS Biol.* **17**, e3000057 (2019).
43. F. Vega-Macaya, C. Manieu, M. Valdivia, M. Mlodzik, P. Olguín, Establishment of the muscle-tendon junction during thorax morphogenesis in *Drosophila* requires the Rho-Kinase. *Genetics* **204**, 1139–1149 (2016).
44. M. Y. Noh *et al.*, Two major cuticular proteins are required for assembly of horizontal laminae and vertical pore canals in rigid cuticle of *Tribolium castaneum. Insect Biochem. Mol. Biol.* **53**, 22–29 (2014).
45. S. Jasrapuria, C. A. Specht, K. J. Kramer, R. W. Beeman, S. Muthukrishnan, Gene families of cuticular proteins analogous to peritrophins (CPAPs) in *Tribolium castaneum* have diverse functions. *PLoS One* **7**, e49844 (2012).
46. M. M. Mogensen, J. B. Tucker, Evidence for microtubule nucleation at plasma membrane-associated sites in *Drosophila. J. Cell Sci.* **88**, 95–107 (1987).
47. S. Li Zheng, J. G. Adams, A. D. Chisholm, Form and function of the apical extracellular matrix: New insights from *Caenorhabditis elegans*, *Drosophila melanogaster*, and the vertebrate inner ear. *Fac Rev* **9**, 27 (2020).
48. Y. Qu, I. Hahn, S. E. Webb, S. P. Pearce, A. Prokop, Periodic actin structures in neuronal axons are required to maintain microtubules. *Mol. Biol. Cell* **28**, 296–308 (2017).
49. Z. Takács *et al.*, The spectraplakin Short stop is an essential microtubule regulator involved in epithelial closure in *Drosophila. J. Cell Sci.* **130**, 712–724 (2017).
50. A. Subramanian *et al.*, Shortstop recruits EB1/APC1 and promotes microtubule assembly at the muscle-tendon junction. *Curr. Biol.* **13**, 1086–1095 (2003).
51. S. Yamada, D. Keyser, Cuticle formation of the adductor muscle attachment in a podocopid ostracod, as an example of a calcified arthropod. *Paleontol. Res.* **13**, 103–118 (2009).
52. A. Jaźwińska, C. Ribeiro, M. Affolter, Epithelial tube morphogenesis during *Drosophila* tracheal development requires Piopio, a luminal ZP protein. *Nat. Cell Biol.* **5**, 895–901 (2003).
53. N. L. Daly, M. J. Scanlon, J. T. Djordjevic, P. A. Kroon, R. Smith, Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6334–6338 (1995).