Chitin deacetylases are necessary for insect femur muscle attachment and mobility

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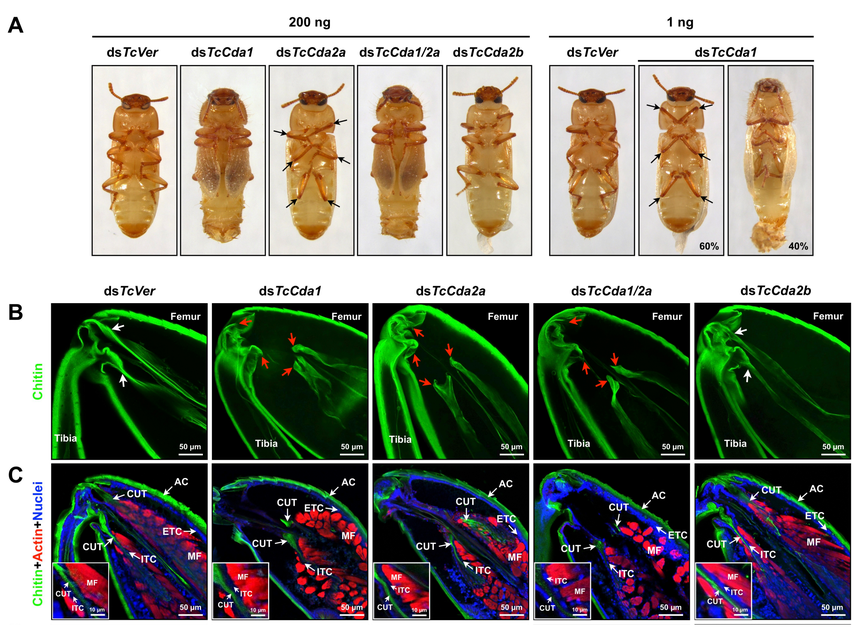
Introduction

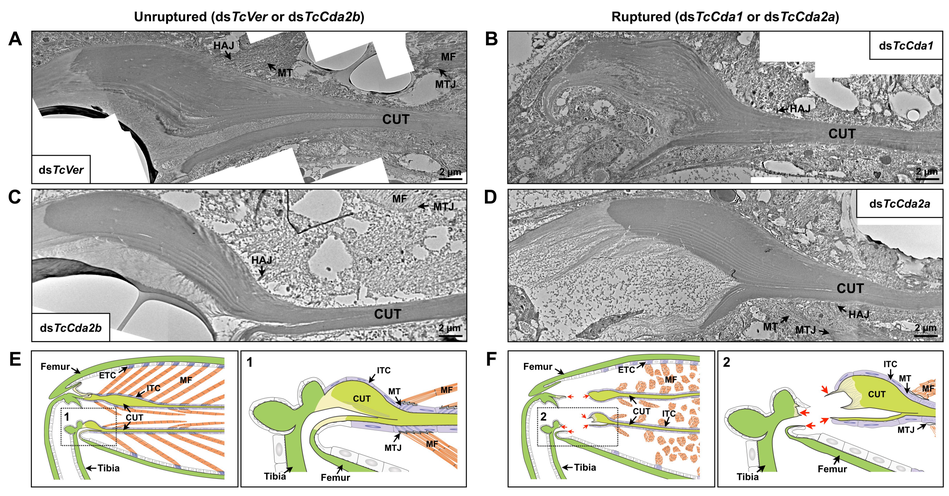
Chitin deacetylases (CDAs) plays critical roles in structural integrity and organization of cuticle in insects. In this study, we reveal a novel function for group I CDAs in insect locomotion and muscle attachment using RNAi approach targeted towards specific CDA isoforms in the red flour beetle, *Tribolium castaneum*. Muscle attachment sites (MASs) in insects and other arthropods involve specialized epithelial cells called tendon cells that adhere to the apical extracellular matrices containing chitin. Depletion of TcCDA1 or the alternatively spliced TcCDA2 isoform, TcCDA2a, causes internal tendon cuticle breakage at the femur-tibia joint, muscle detachment from both internal and external tendon cells, and defective locomotion. These results suggest that 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.

Morphology of insects, limb tendons and muscles of pharate adults after RNAi for *TcCdas*



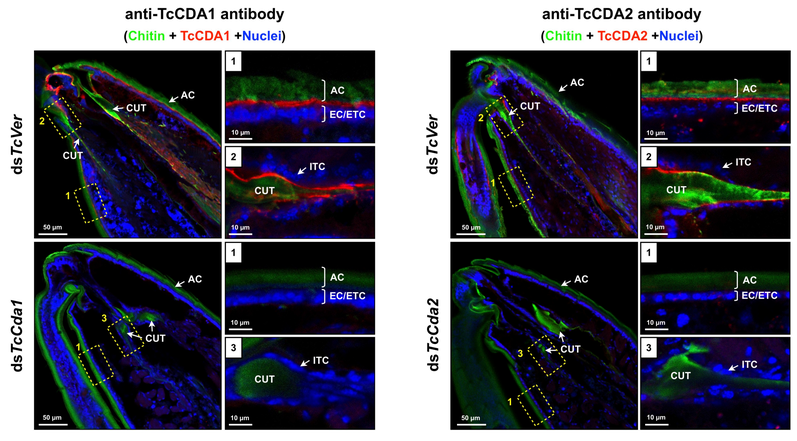
dsRNA (200 ng per insect) for *TcVer, TcCda1*, *TcCda2a*, *TcCda1/2a* or *TcCda2b* was injected into day 0 pupae. (A) ds*TcCda1*- or ds*TcCda1/2a*-treated pupae failed adult eclosion and died without shedding their pupal exuviae, while ds*TcCda2a* and ds*TcCda2b* pupae did molt to adults. However, the resulting ds*TcCda2a* adults, but not ds*TcCda2b* adults, were unable to move their femur-tibia joints (arrows in left panel). Similarly, ~60% of the pupae treated with 1 ng of ds*TcCda1* were able to eclose, but the resulting adults exhibited impaired leg movement (arrows in right panel). (B) Legs dissected from the dsRNA-treated day 5 pupae were incubated with 10 M NaOH, followed by staining for chitin with FITC-conjugated chitin-binding probe (FITC-CBD, green). Unlike ds*TcVer* control and *TcCda2b* animals, which exhibited intact internal tendon cuticle (white arrows), ds*TcCda1*, ds*TcCda2a* and ds*TcCda1/2a* (co-injection) animals showed internal tendon cuticle breakage at the femur-tibia leg joint (red arrows). (C) Cryosections of the legs from dsRNA-treated day 5 pupae were incubated with FITC-CBD, Alexa Fluor 546-phalloidin and TO-PRO-3 to stain chitin (green), actin (red) and nuclei (blue), respectively. ds*TcVer* control and *TcCda2b* animals showed steretched femur muscles, in which one end was firmly anchored to the internal tendon cell (ITC) basal matrix and the other end adhered to the external tendon cell (ETC). In contrast, most of the femur muscles in ds*TcCda1*, ds*TcCda2a* or ds*TcCda1/2a* animals were detached from both the internal and external tendon cells, indicating an absence of attachment at either end. A representative region of muscle fiber (MF) attachment to internal tendon cell is enlarged in the insets. AC, adult cuticle; CUT, internal tendon cuticle.

Morphology and break-point of internal tendon cuticles of femurs after RNAi



Ultrastructure of the internal tendon cuticles (CUT) of the femurs from day 5 pupae that had been injected with ds*TcVer,* ds*TcCda1*, ds*TcCda2a* or ds*TcCda2b* into day 0 pupae. Unlike ds*TcVer*-control or ds*TcCda2b* animals (A and C), in ds*TcCda1* and ds*TcCda2a* insects, the internal tendon cuticle had ruptured near the femur-tibia joint, and the chitinous laminae were partially unraveled (B and D), suggesting that this joint had become mechanically vulnerable due to TcCDA1 or TcCDA2a deficiency. Schematic diagram of the femur segments of dsRNA-treated day 5 pupae illustrating the unruptured (E) and ruptured (F) internal tendon cuticles of femurs and detachment of femur muscle fibers (MF) from external tendon cells (ETC) and internal tendon cells (ITC). One of the internal tendon cuticle junctions between the tibial and femur segments (dotted line boxes 1 and 2 in panels E and F) is enlarged in right panels. The internal tendon cuticle breaks between femur and tibial leg segments are indicated by red arrows in panel F. MT, microtubule; MTJ, myotendinous junction; HAJ, hemi-adherens junctions.

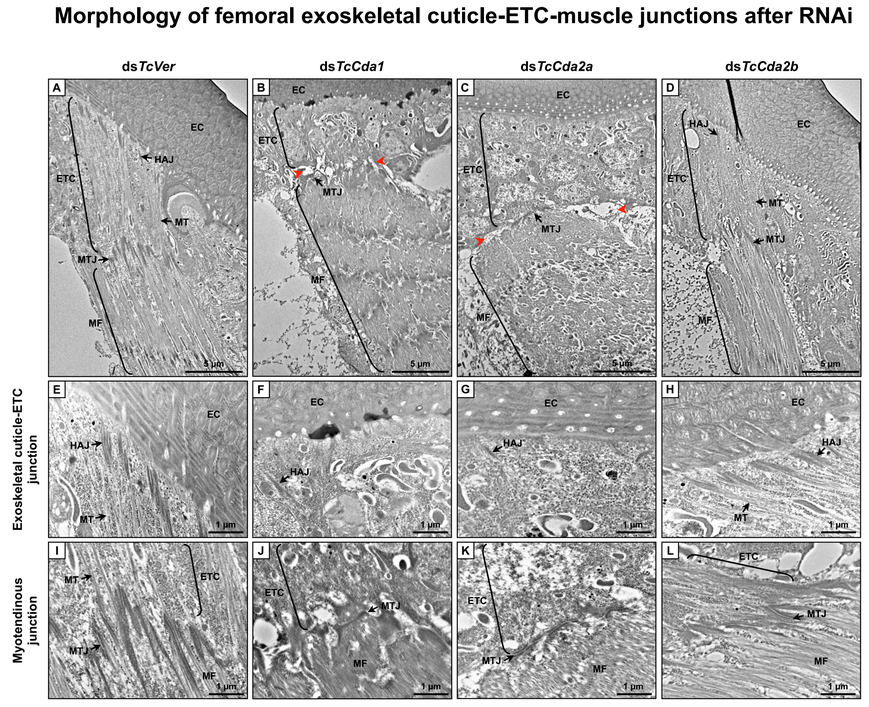
Localization of TcCDA1 and TcCDA2 in leg internal tendon cuticle



Cryosections of legs from day 5 pupae treated with ds*TcVer*, ds*TcCda1* or ds*TcCda2* were incubated with anti-TcCDA1 (left panels) or anti-TcCDA2 antibodies (right panels), which were then detected by secondary Alexa Fluor 546-conjugated goat anti-rabbit IgG (red). Cuticular chitin and nuclei were stained with FITC-CBD (green) and TO-PRO-3 (blue), respectively. Both TcCDA1 and TcCDA2 proteins were localized near the apical membrane of both the internal tendon cells (ITC) and external tendon cells (ETC) underlying the cuticle as a narrow band, suggestive of membrane association and/or localization in the assembly zone of the cuticle. The exoskeletal cuticle (box 1), internal tendon cuticle (CUT) junction between femur and tibial segments (box 2) and tips of the ruptured internal tendon cuticle (box 3) were enlarged. AC, adult cuticle; EC/ETC, epidermal cell/external tendon cell.

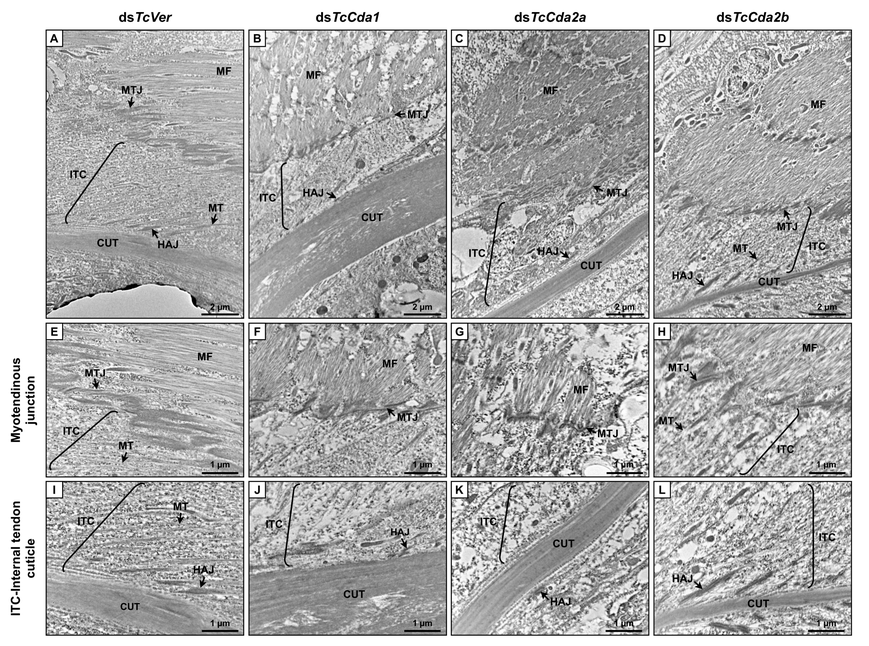
Mechanical links between exoskeletal cuticle and internal tendon cuticle

Morphology of femoral exoskeletal cuticle-ETC-muscle junctions after RNAi



(A-D) Ultra-thin 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-external tendon cell (ETC)-muscle junctions by TEM. The external tendon cells of ds*TcVer* control and ds*TcCda2b* femurs had parallel microtubules (MT) that connected the electron dense hemi-adherens junctions (HAJ) and myotendinous junctions (MTJ), found at either the apical or basal ends of the tendon cells (E, H, I and L). However, in ds*TcCda1* and ds*TcCda2a* femurs, both MTs and HAJs were missing or greatly diminished (F and G). In addition, the MTJs of those femurs lacked the characteristic zigzag membrane interdigitations (J and K) and exhibited gaps between external tendon cells and muscle cells (red arrowheads in B and C) where MTJs could be discerned, they were flat, resulting in greatly decreased contact surface areas at the boundaries of the external tendon cells and muscle cells (J and K). EC, exoskeletal cuticle; MF, muscle fiber.

Morphology of femoral muscle-ITC-internal tendon cuticle junctions after RNAi



(A-D) Legs were dissected from day 5 pupae treated with ds*TcVer*, ds*TcCda1*, dsT*cCda2a* or ds*TcCda2b* for analysis of ultrastructure of the muscle-internal tendon cell (ITC)-internal tendon cuticle (CUT) junctions by TEM. Larger magnifications of the MTJ between muscle fiber and ITC (E-H) and the ITC-internal tendon cuticle junctions (I-L). The extensive membrane interdigitations between myotubes and ITCs present in ds*TcVer* control and ds*TcCda2b* femurs (E and H) were replaced by flat and discontinuous connections with a greatly reduced contact surface area between the two cell types in ds*TcCda1* and ds*TcCda2a* femurs (F and G). Furthermore, the extensive microtubules connecting the apical and basal HAJs and MTJs of ITCs were either absent or deficient the muscle fibers (MF) were noticeably less stretched in the later femurs (J and K) compared to the former ones (I and L).

Conclusions

In this work, we demonstrate that loss of functions of group I CDAs from *T. castaneum*, TcCDA1 and TcCDA2a, causes breakage of the chitinous internal tendon cuticle at the femur-tibia joint, muscle detachment from both internal and external tendon cells, which lead to defective locomotion.
Our studies reveal a previously unrecognized role of insect CDAs in musculoskeletal connectivity, maintenance of tendon cell microtubule integrity, muscle force transmission, limb movement and locomotion.
All of these results indicate 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 both internal and external tendon cells.

Reference

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Acknowledgment

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