Supporting Information

Chaudhari et al. 10.1073/pnas.1112288108

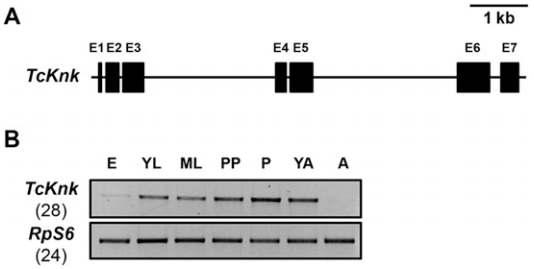

Fig. S1. Developmental stage-specific expression pattern of the *Tribolium castaneum Knickkopf (TcKnk)* was determined by RT-PCR. (*A*) Schematic diagram of the exon–intron organization of the *TcKnk* gene. The exon–intron organization of the *TcKnk* gene was determined by sequence comparison between genomic sequence and the full-length cDNA sequence containing 5′ and 3′ UTR regions. This gene is composed of seven exons and encodes a 75-kDa protein that shares 60% amino acid sequence identity with *Drosophila melanogaster* Knk. Black solid boxes indicate exons, and lines indicate introns. (*B*) Expression of *TcKnk* during insect development. Total RNA was prepared from eggs (E), young larvae (YL), mature last-instar larvae (ML), pharate pupae (PP), pupae (P), young adult (YA), and adult (A) stages. cDNAs synthesized from total RNAs with oligo$(dT)_{20}$ primers and reverse transcriptase were used as templates for RT-PCR (28 cycles) using gene-specific primer pairs. *RpS6* was used as an internal loading control (24 cycles).

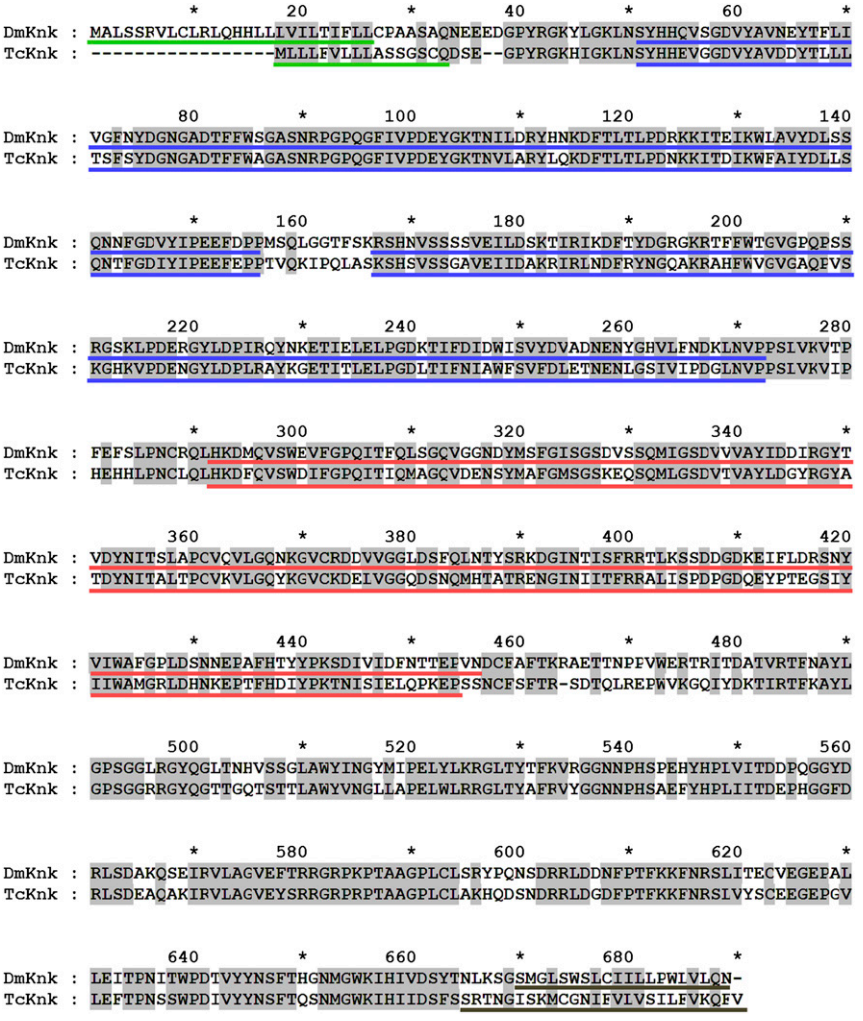

Fig. S2. Amino acid sequence alignment of TcKnk and *D. melanogaster* Knk (DmKnk). Gray shading of amino acid residues indicates identity. Underlines of different colors indicate different domains: green, leader peptide; blue, DM13 domains; red, dopamine monooxygenase N-terminal like (DOMON) domain; black, GPI anchor-specifying sequence.

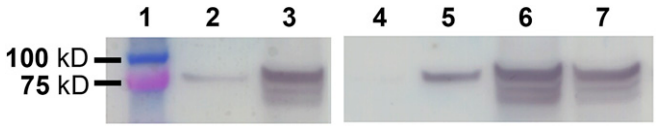

Fig. S3. TcKnk is a GPI-anchored, membrane-bound protein. Recombinant TcKnk protein expressed in Hi-5 cells infected with a recombinant baculovirus containing the ORF of TcKnk is shown. After 72 h of infection, the medium was removed, and fresh medium was added along with 100 μL of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* (7.89 units/mg) for 4 h. The proteins in the medium and cell pellet were subjected to Western blot analysis with an anti-Knk antiserum. Lane 1, size marker; lane 2, medium from TcKnk-expressing Hi-5 cells after 72 h of infection; and lane 3, cell pellet from TcKnk-expressing Hi-5 cells at 72 h after infection. For lanes 4–7, old medium was removed and replaced with fresh medium with or without added PI-PLC. Lane 4, medium from TcKnk-expressing Hi-5 cells after mock treatment for 4 h without PI-PLC; lane 5, medium from TcKnk-expressing Hi-5 cells at 4 h after PI-PLC treatment; lane 6, TcKnk-expressing Hi-5 cell pellet without PI-PLC treatment; and lane 7, cell pellet from TcKnk-expressing Hi-5 cells after 4 h of PI-PLC treatment. TcKnk was found predominantly in the cell pellet fraction (lane 3 versus lane 2), and some of it was released to the medium after 4 h of PI-PLC treatment (compare lane 5 with lanes 4 and 7). The two lower immunoreactive bands probably represent TcKnk protein without GPA anchor and/or unprocessed forms.

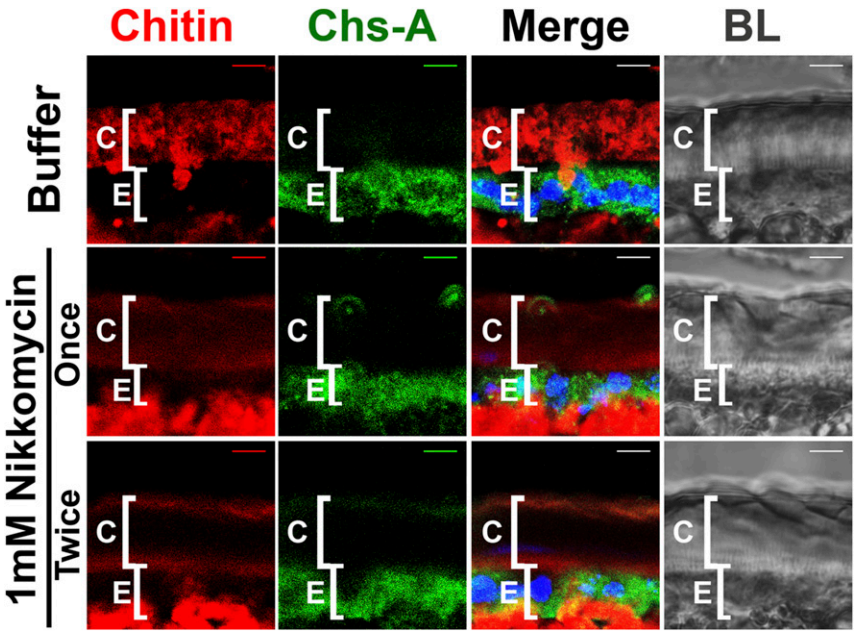

Fig. S4. Localization of *T. castaneum* chitin synthase A (TcChs-A) in elytra of pharate adults after nikkomycin treatment. Cellular distribution of TcChs-A (green) in insects injected once or twice with 0.5 μL of 1 mM nikkomycin (a chitin synthase inhibitor) was not changed in comparison with buffer-injected insects after analysis by confocal microscopy (as described in Fig. 3). Red, rhodamine-conjugated chitin-binding probe; C, cuticle; E, epithelial cells. (Scale bars: 5 μm.) The red staining below the epidermis is nonspecific, whereas the staining above the epidermis is chitin-specific.

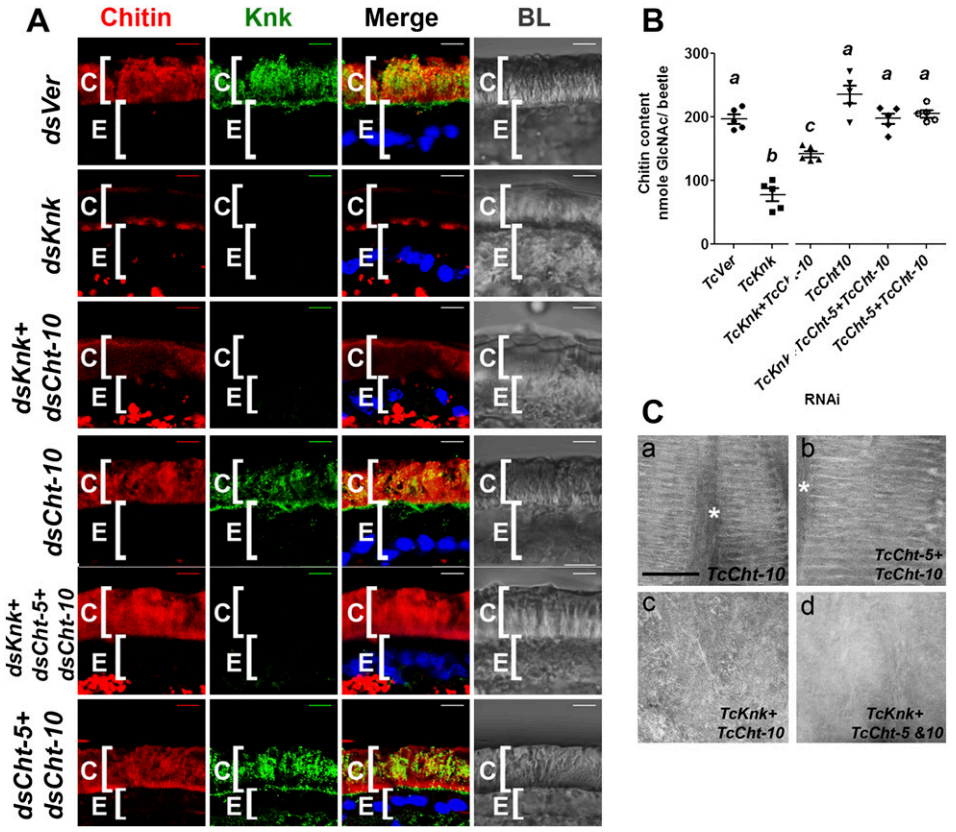

Fig. S5. Knk protects procuticular chitin from chitinases. (*A*) Immunostaining of cryosections of lateral abdominal body wall from control (*dsVer*) pharate adults or after treatment with the indicated combinations of dsRNAs for *TcKnk* (*dsKnk*), *TcCht-10* (*dsCht-10*), and *TcCht-5* (*dsCht-5*). Chitin, red; Knk, green; DAPI, blue; C, cuticle; E, epithelial cells. (Scale bars: 5 μm.) (*B*) Analysis of total chitin content by a modified Morgan–Elson assay. Data are reported as mean ± SE ($n = 5$ each). Statistical significance was computed with Student's *t* test. Means identified by different letters (a, b, and c) are significantly different at $P < 0.05$. (*C*) Ultrastructure of pharate adult elytral cuticle. Cuticle after *TcCht-10* single (*a*) or *TcCht-5 and TcCht-10* double (*b*) dsRNA treatment is normal and indistinguishable from *T. castaneum Vermilion* (*TcVer*) dsRNA-treated samples (Fig. 4*C*). The elytral cuticle of pharate adults treated with dsRNA for *TcKnk* and *TcCht-10* (*c*), or *TcCht-5* and *TcCht-10* along with dsRNA for *TcKnk* (*d*) does not restore the laminar organization of chitin. (Scale bar: 500 nm.)

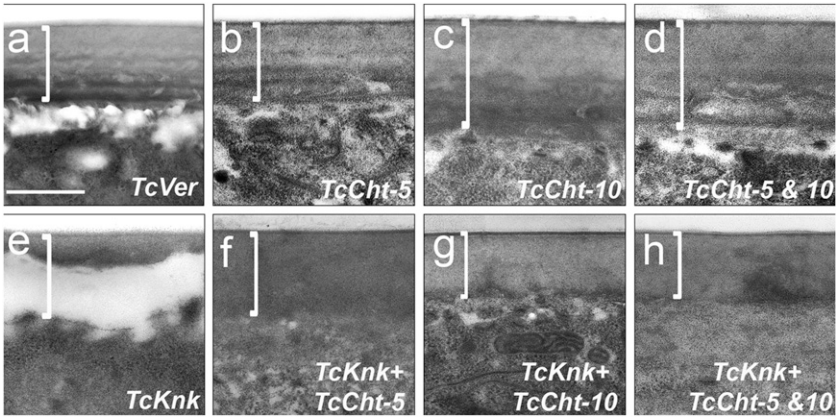

Fig. S6. Laminar organization of the ventral body wall cuticle of pharate adults depends on TcKnk, TcCht-5, and TcCht-10. The ventral body wall cuticle (bracket) of control animals (*TcVer*) is arranged in horizontal sheets presumably made up of chitin (*A*). This laminar organization is not perturbed in the respective cuticles of animals treated with dsRNAs for *TcCht-5* (*B*), *TcCht-10* (*C*), or *TcCht-5* and *TcCht-10* (*D*). By contrast, upon *TcKnk* knockdown in the ventral body wall cuticle, the laminae are absent (*E*). Simultaneous knockdown of *TcKnk* transcripts along with *TcCht-5* (*F*), *TcCht-10* (*G*), or *TcCht-5* and *TcCht-10* (*H*) does not restore laminar structure even though chitin levels are restored (Fig. 4*C* and Fig. S5*C*). (Scale bar: 500 nm.)

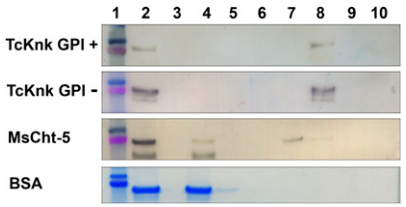

Fig. S7. Colloidal chitin-binding assay of recombinant TcKnk, chitinase, and BSA. TcKnk with (TcKnk GPI+) and without (TcKnk GPI–) GPI anchor was prepared as described in *Materials and Methods*. TcKnk was either extracted from the cell pellet with the Mem-PER Kit (TcKnk GPI+) or released by treatment with PI-PLC (TcKnk GPI–). *Manduca sexta* chitinase 5 (MsCht-5) recombinant protein was purified as described previously (1). Proteins used for the chitin-binding assay are indicated on the left. Lane 1, size markers; lane 2, protein sample as indicated on the left; lane 3, colloidal chitin used for the binding assay; lane 4, flow-through fraction; lane 5, 10 mM sodium phosphate buffer (pH 8.0) wash; lane 6, 10 mM sodium phosphate buffer containing 1 M NaCl (pH 8.0) wash; lane 7, 1% Calcofluor eluate; lane 8, protein eluted by boiling the colloidal chitin with bound proteins for 10 min in 50 μL of 1× SDS/PAGE sample buffer; lane 9, negative control (uninfected Hi-5 cells after PI-PLC treatment or proteins extracted from them by Mem-PER Kit); and lane 10, proteinase inhibitor mixture that was added to all buffers to minimize proteolysis. The samples were run on a SDS/4–12% PAGE gradient gel, and the presence of TcKnk and MsCht-5 in each fraction was detected by Western blot analysis using the horseradish peroxidase-conjugated secondary antibody detection system (Bio-Rad). BSA (Sigma) was detected by Coomassie blue staining. The absence in the Calcofluor-eluate fraction of TcKnk protein and its presence in the SDS-boiled fraction demonstrated strong affinity of TcKnk for colloidal chitin. MsCht-5 was used as a positive control for chitin binding. MsCht-5, which has one chitin-binding domain, had a lower affinity for colloidal chitin than TcKnk did because most of this protein was released into the supernatant by 1% Calcofluor. A truncated form of this protein, which lacks the C-terminal chitin-binding domain, does not bind to chitin and is recovered in the flow-through fraction (the lower band in the MsCht-5 gel). BSA was used as a negative control for nonspecific binding of proteins to chitin.

1. Arakane Y, Zhu Q, Matsumiya M, Muthukrishnan S, Kramer KJ (2003) Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem Mol Biol* 33:631–648.

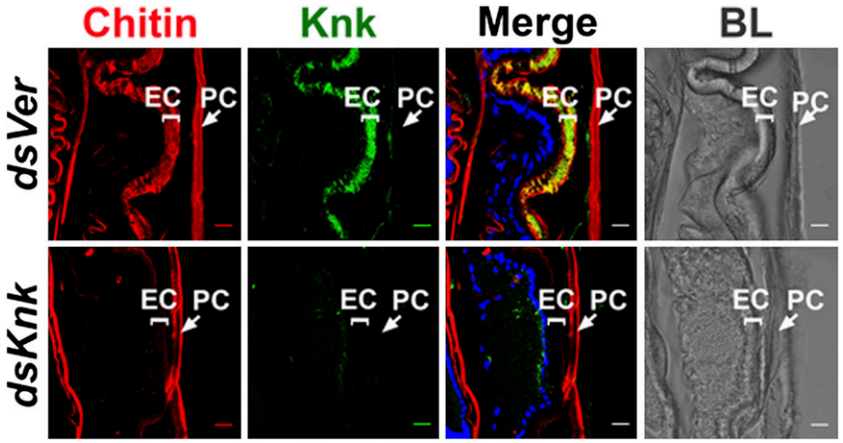

Fig. S8. TcKnk is preferentially detected in the new cuticle. Chitin staining (red) of cryosections of pharate adult insects containing both the old pupal cuticle (PC) and the newly synthesized elytral cuticle (EC). TcKnk (green) is only detected in the new elytral cuticle. Nuclei are stained with DAPI (blue). (Scale bars: 5 μm.) Insects subjected to treatment with dsRNA for *TcKnk* were included to demonstrate the specificity of the Knk antibody.

Table S1. Primers used for dsRNA synthesis

dsRNA	Direction	Sequence (5′ → 3′)	Nucleotide positions	dsRNA length, bp
1	Forward	TAATACGACTCACTATAGGGTGGCCAAACATCCACG	1,449–1,900	451
	Reverse	TAATACGACTCACTATAGGGTCCATGTTTGACTGCG		
2	Forward	TAATACGACTCACTATAGGGTTGGGACAATATAAAGGTG	1,036–1,201	165
	Reverse	TAATACGACTCACTATAGGGTAAATTGAGCCCTCGG		