

THE MOLECULAR MECHANISMS OF KNICKKOPF AND RETROACTIVE  
PROTEINS IN ORGANIZATION AND PROTECTION OF CHITIN IN  
THE NEWLY SYNTHESIZED INSECT EXOSKELETON

by

SUJATA SURESH CHAUDHARI

B.Sc., University of Pune, India, 2002

M.Sc., University of Pune, India, 2004

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submitted in partial fulfillment of the requirements for the degree

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Graduate Biochemistry Group

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## Abstract

In order to grow and develop, insects must undergo a process of molting, wherein the old cuticle is replaced with a new one. A thin envelope layer has been predicted to act as a physical barrier between molting fluid chitinases and the site of new chitin synthesis ensuring selective protection of newly synthesized chitin. The factors that help the new exoskeleton withstand the deleterious effects of chitinolytic enzymes remain poorly understood.

In the current study a mechanistic role for two proteins, Knickkopf (Knk) and Retroactive (Rtv), was explored in organization and protection of the newly synthesized procuticular chitin. Our study demonstrated colocalization of molting fluid chitinases (chitinase-5) with chitin in *T. castaneum* pharate adult elytral cuticle. Presence of chitinases in the new cuticle, disproved the old theory of the envelope being a protective barrier against chitinases. Confocal and transmission electron microscopic imaging of *T. castaneum* pharate adult elytral cuticle suggested that Knk protein selectively colocalizes with chitin in the new procuticle, organizes chitin into laminae and protects it from the activity of molting fluid chitinases. Down-regulation of *Knk* expression resulted in reduction of procuticular chitin, disruption of the laminar architecture of the procuticle and severe molting defects that are ultimately lethal at all stages of insect growth.

The presence and activity of Rtv protein ensures the trafficking of Knk into the procuticle. Down regulation of *Rtv* transcripts showed molting defects and a significant decrease in chitin content similar to those following *Knk* dsRNA treatment. Confocal microscopic analysis revealed an essential role for Rtv in proper trafficking of Knk from epithelial cells to within the newly synthesized procuticle. Once released into the procuticle, Knk organizes and protects chitin from chitinases. The conservation of Knk and Rtv in all insect species suggests a critical role for these proteins in maintenance and protection of chitin in the insect exoskeleton.

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Approved by:

Major Professor

Dr. Subbaratnam Muthukrishnan

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# Table of Contents

<b>List of Figures.....</b>	<b>x</b>
<b>List of Tables .....</b>	<b>xii</b>
<b>Acknowledgements.....</b>	<b>xiii</b>
<b>Chapter 1 - Introduction.....</b>	<b>1</b>
Insect Cuticle .....	2
Chitin synthesis.....	4
Chitin organization in the cuticle .....	5
Proteins associated with chitin in the cuticle .....	5
Molting in insects.....	6
Cuticulin layer.....	10
Outline of this thesis.....	12
References .....	13
<b>Chapter 2 - The Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton .....</b>	<b>20</b>
Abstract.....	21
Introduction.....	22
Materials and Methods .....	23
<i>Tribolium</i> strains .....	23
Identification, cloning and phylogenetic analysis of <i>TcKnk</i> .....	23
Determination of expression profile of TcKnk .....	23
dsRNA synthesis .....	24
Injection of dsRNA into <i>T. castaneum</i> .....	24
Measurement of transcripts for <i>TcKnk</i> after dsRNA treatment .....	25
Expression of TcKnk .....	25
PI-Phospholipase-C (PLC) treatment and western blot analysis .....	25
Immunohistochemistry and confocal analysis .....	26
Chitin content analysis.....	26
Colloidal chitin-binding assay .....	27

Transmission electron microscopy .....	27
Results and Discussion .....	28
Co-localization of chitinase with chitin in the newly synthesized cuticle.....	28
Effect of <i>TcKnk</i> on molting and chitin content. ....	30
Procuticular chitin associates with TcKnk and dictates its cellular distribution.....	33
TcKnk protects cuticular chitin from degradation by chitinases and organizes chitin into laminae.....	36
Knk is widely distributed in invertebrates with chitinous extracellular matrices. ....	42
Concluding remarks .....	43
References .....	45
<b>Chapter 3 - Retroactive promotes trafficking of the chitin-binding protein Knickkopf into the procuticle of <i>Tribolium castaneum</i> .....</b>	<b>48</b>
Abstract.....	49
Introduction.....	50
Materials and methods.....	52
Insect cultures.....	52
Identification of the <i>TcRtv</i> gene in the <i>T. castaneum</i> genome database.....	52
Cloning and sequencing of a <i>TcRtv</i> cDNA.....	52
Phylogenetic analysis of TcRtv.....	52
Determination of expression profiles of TcRtv.....	53
RNA interference studies.....	53
Expression of recombinant TcRtv.....	54
Immunohistochemistry .....	54
Chitin content analysis.....	55
Transmission electron microscopic (TEM) analysis .....	55
Results .....	55
TcRtv is a conserved protein required for insect molting.....	55
TcRtv is important for the maintenance of procuticular chitin .....	58
TcRtv prevents chitinase-mediated degradation of procuticular chitin.....	59
TcRtv affects the ultrastructure of the procuticle.....	60
TcRtv is essential for the procuticular localization of TcKnk .....	62

Discussion.....	64
A role for TcRtv in new cuticle maintenance during molting .....	64
How does TcRtv protein affect the movement of TcKnk to the procuticle? .....	66
TcRtv may be a member of the Ly-6 superfamily of proteins .....	67
References .....	69
<b>Chapter 4 - Chitin synthase A, Knickkopf and Retroactive are proteins required for embryonic serosal cuticle formation in the red flour beetle, <i>Tribolium castaneum</i> .....</b>	<b>72</b>
Abstract.....	73
Introduction.....	74
Material and methods .....	75
Insect cultures.....	75
RNA interference studies.....	75
Dissection of ovaries and testes .....	76
Chitin content analysis of eggs .....	76
Transmission electron microscopic analysis (TEM) .....	76
Results and Discussion.....	77
References .....	83
<b>Chapter 5 - Characterization and functional analysis of two <i>Knk</i>-like genes in the red flour beetle, <i>Tribolium castaneum</i> .....</b>	<b>86</b>
Abstract.....	87
Introduction.....	88
Material and methods .....	89
Insect cultures.....	89
Identification of <i>TcKnk</i> -like genes in the <i>T. castaneum</i> genome database.....	89
Identification of <i>TcKnk</i> -like genes in insect genomes.....	89
Cloning and sequencing of cDNAs for <i>TcKnk</i> -like genes.....	89
Phylogenetic analysis of TcKnk-like proteins .....	91
Developmental and tissue-specific expression profile for <i>TcKnk</i> -like genes .....	91
RNA interference studies.....	91
Parental RNAi .....	92
Northern blot for detection of <i>TcKnk3</i> transcripts .....	92



Chitin content analysis.....	92
Transmission electron microscopic (TEM) analysis .....	93
Results .....	93
Identification, cloning and sequencing of <i>TcKnk</i> -like genes from <i>T. castaneum</i> genome ....	93
Domain organization and phylogenetic analysis of TcKnk2 and TcKnk3 proteins .....	95
Knk-like proteins are present in several orders of insects .....	96
Developmental stage and tissue-specific expression profiles of <i>TcKnk2</i> and <i>TcKnk3</i> .....	96
<i>TcKnk2</i> contributes to pupal-adult molt of <i>T. castaneum</i> development .....	99
RNAi reveals that the 3'-half of the <i>TcKnk3</i> gene may be transcribed to yield a shorter transcript essential for adult morphogenesis. ....	100
Detection of <i>TcKnk3</i> -3' transcripts by northern blot analysis .....	104
Role of <i>TcKnk</i> -like genes in embryonic development of <i>T. castaneum</i> .....	107
TcKnk2 and TcKnk3 are required for chitin maintenance and integrity of procuticular chitin in <i>T. castaneum</i> .....	107
Discussion.....	111
References .....	114
<b>Chapter 6 - Summary and Discussion .....</b>	<b>117</b>
General conclusions .....	118
Future Directions.....	121
References .....	123
<b>Appendix A - Permission to release copyrighted material .....</b>	<b>125</b>
<b>Appendix B - Immunohistochemistry controls .....</b>	<b>126</b>
<b>Appendix C - Amino acid alignment for TcKnk, TcKnk2 and TcKnk3 .....</b>	<b>127</b>

## List of Figures

Figure 1.1 Structure of insect cuticle. ....	2
Figure 1.2 Transmission electron microscopic (TEM) analysis of <i>T. castaneum</i> larval cuticle. ....	3
Figure 1.3 Classical model of molting in insects.....	9
Figure 2.1 Chitinase co-localizes with chitin in the newly-synthesized cuticle. ....	28
Figure 2.2 Exon–intron organization and developmental stage-specific expression pattern of <i>TcKnk</i> .....	29
Figure 2.3 Amino acid sequence alignment of TcKnk and DmKnk.....	31
Figure 2.4 Domain organization, expression and role of TcKnk in maintenance of cuticular chitin. ....	32
Figure 2.5 TcKnk is a GPI-anchored, membrane-bound protein. ....	33
Figure 2.6 TcKnk is mislocalized following disruption of chitin synthesis. ....	34
Figure 2.7 Localization of TcChs-A in elytra of pharate adults after nikkomycin treatment. ....	35
Figure 2.8 TcKnk aids laminar organization of the procuticular chitin and prevents its degradation by molting fluid chitinases.....	37
Figure 2.9 Knk protects procuticular chitin from chitinases. ....	38
Figure 2.10 Laminar organization of the ventral body wall cuticle of pharate adults depends on TcKnk, TcCht-5 and TcCht-10. ....	39
Figure 2.11 Colloidal chitin-binding assay of recombinant TcKnk, chitinase and bovine serum albumin. ....	40
Figure 2.12 TcKnk is preferentially detected in the new cuticle. ....	42
Figure 2.13 Phylogenetic analysis of Knickkopf-like proteins from different taxa. ....	43
Figure 2.14 Model of chitin deposition and degradation in the composite insect cuticle during molting.....	44
Figure 3.1 Exon-intron and domain organization of TcRtv. ....	56
Figure 3.2 TcRtv is a conserved protein required for insect molting. ....	57
Figure 3.3 TcRtv is important for the sustenance of procuticular chitin.....	58
Figure 3.4 Localization of TcChs-A in insects treated with dsRNA for <i>TcVer</i> and <i>TcRtv</i> . ....	60
Figure 3.5 TcRtv prevents chitinase-mediated degradation of procuticular chitin.....	61

Figure 3.6 TcRtv influences the localization of TcKnk to the procuticle. ....	63
Figure 3.7 TcRtv may be a Ly-6 family protein. ....	67
Figure 4.1 Effect of <i>TcChs-A</i> , <i>TcKnk</i> and <i>TcRtv</i> dsRNA-treatment on ovarian development. ....	78
Figure 4.2 Effect of dsRNA-treatment for <i>TcChs-A</i> and <i>TcKnk</i> into adult females of <i>T. castaneum</i> on egg hatch. ....	79
Figure 4.3 Effect of parental RNAi on <i>T. castaneum</i> embryonic chitin content. ....	80
Figure 4.4 Transmission electron microscopic (TEM) analysis of serosal cuticle. ....	82
Figure 5.1 Schematic diagram of the exon–intron organizations and domain architecture of the putative <i>Tribolium castaneum</i> <i>Knk</i> -like genes and proteins. ....	94
Figure 5.2 Phylogenetic analysis of insect TcKnk2 and TcKnk3. ....	97
Figure 5.3 Developmental stage- and tissue-specific expression of <i>TcKnk</i> -like genes by RT-PCR. ....	98
Figure 5.4 Effect <i>TcKnk2</i> dsRNA-treatment on the development of <i>T. castaneum</i> . ....	100
Figure 5.5 Effect of <i>TcKnk3</i> dsRNA-treatment on the development of <i>T. castaneum</i> . ....	102
Figure 5.6 Specificity of dsRNA-mediated down-regulation of <i>TcKnk3</i> transcripts. ....	103
Figure 5.7 Northern blot analysis of <i>TcKnk3</i> transcripts using <sup>32</sup> P-labeled 5'-terminal and 3'-terminal probes. ....	105
Figure 5.8 Sequence for <i>TcKnk3</i> -3' transcript. ....	106
Figure 5.9 Effect of <i>TcKnk2</i> and <i>TcKnk3</i> dsRNA treatment on ovaries and cuticular chitin content of <i>T. castaneum</i> . ....	108
Figure 5.10 Effect of TcKnk3 dsRNA-treatment on embryonic chitin content. ....	109
Figure 5.11 Effect of <i>TcKnk2</i> and <i>TcKnk3</i> dsRNA treatment on procuticle structure of <i>T. castaneum</i> . ....	110

## List of Tables

Table 2.1 Primers used for dsRNA synthesis. ....	24
Table 3.1 Primer sequences for generation of dsRNAs. ....	53
Table 4.1 Primer sequences for generation of dsRNAs. ....	76
Table 4.2 Effect of parental RNAi on oviposition and egg hatching in <i>T. castaneum</i> . ....	77
Table 5.1 Primer sequences .....	90

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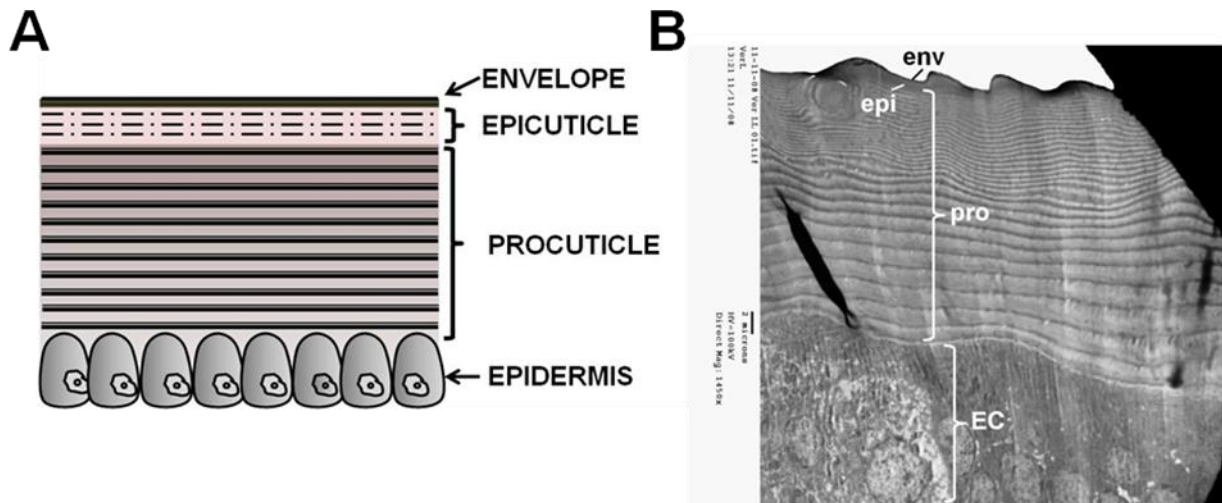
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# **Chapter 1 - Introduction**

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## Insect Cuticle

The cuticle or exoskeleton is the primary physical barrier that protects insects against infection, injury and desiccation. Insect cuticle consists of three distinct layers, the outermost waxy envelope (superficial layer), middle protein-rich epicuticle and innermost (just above the epithelial cells) chitin- and proteins-rich procuticle (Fig 1.1). The envelope is the first layer synthesized by epithelial cells during cuticle development, which covers the whole surface of the insect body except some sensory areas and has been shown to be important for protection, permeability and pigmentation (Locke, 2001). Lipid composition of isolated waxy layers has been determined by gas chromatography and mass spectroscopy in some insects indicating the presence of long chain hydrocarbons, esters, alcohol and carboxylic acids in this layer. Lipid composition of the envelope varies depending on the insect species and probably the diet they consume.



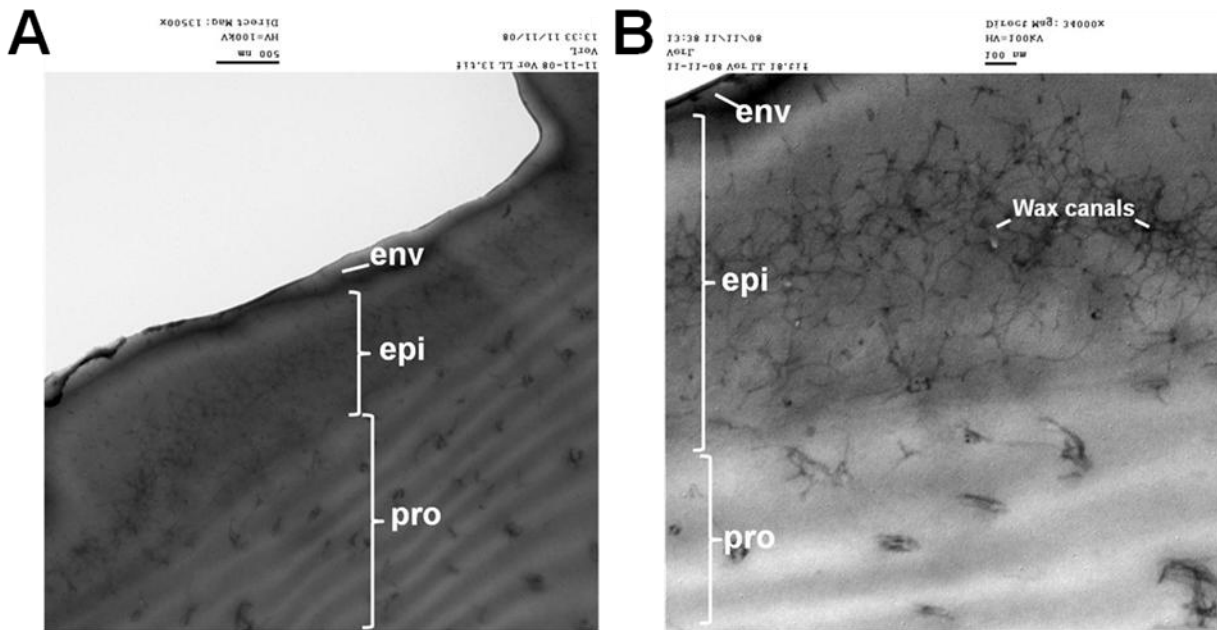
**Figure 1.1 Structure of insect cuticle.**

(A) Diagrammatic representation and (B) Transmission electron microscopic analysis of *T. castaneum* larval cuticle. The cuticle is made up of three distinct layers including the outermost waxy envelope (env), middle protein-rich epicuticle (epi) and the innermost chitin- and protein-rich procuticle (pro). All three layers of chitin are synthesized by the underlying epithelial cells (EC). Scale bar = 2 microns.

Electron microscopic analysis of completely developed insect cuticle indicates the presence of wax canals in the epicuticle that transport wax to the cuticle surface (Fig 1.2). In some insects, these wax canals connect with the pore canals in the procuticle and terminate in the outermost



envelope layer (Locke, 1961). The exact mechanism of transport of lipids from the epithelial cells to the outer envelope in a fully developed cuticle is still unknown. Using histochemical procedures, the presence of esterase activity in the wax canals was demonstrated, confirming the notion that lipids are secreted by wax canals (Andersen, 1979; Locke, 1961; Wigglesworth, 1947a). The middle epicuticle layer is mainly made up of highly cross-linked proteins and lipids. Though the exact composition of the epicuticle is still a mystery, gold labeling and electron microscopic analysis indicates the absence of chitin in the epicuticle (Locke, 2001; Sass et al., 1994).



**Figure 1.2 Transmission electron microscopic (TEM) analysis of *T. castaneum* larval cuticle.**

(A-B) TEM showing magnified view of *T. castaneum* larval cuticle. Envelope, epicuticle and procuticle layers are distinct. Outermost envelope and middle epicuticle are electron dense. Epicuticle shows the presence of wax canals that secrete wax to the cuticle surface. Procuticle shows laminar organization of chitin. Scale bar; A=500 nm; B=100 nm.

The innermost procuticle layer above epithelial cells is made up of chitin and proteins. Chitin, a homopolymer of  $\beta$  1-4 linked N-acetyl glucosamine units, is the second most abundant

polymer in nature. It is found in most fungi, nematodes and arthropods (Merzendorfer, 2006). Chitin is not only a major component of the insect procuticle but it is also found in the tracheae, foregut, hindgut, peritrophic matrix and eggshells (Kramer and Muthukrishnan, 2005). X-ray diffraction analysis indicates that chitin occurs naturally in three different forms,  $\alpha$ -,  $\beta$ - and  $\gamma$ -chitin (Cohen, 1987; Jang et al., 2004; Merzendorfer, 2006; Merzendorfer and Zimoch, 2003). The  $\alpha$ -form of chitin exhibits anti-parallel orientation of chitin chains and is abundantly found in the cuticle. The  $\beta$ -form has parallel chains while the  $\gamma$ -form shows combination of two parallel and one anti-parallel chain of chitin. Both the  $\beta$ - and  $\gamma$ - forms are found in squid pens and in cocoons, respectively (Jang et al., 2004; Merzendorfer, 2006; Merzendorfer and Zimoch, 2003). Anti-parallel arrangement of chitin polymers ( $\alpha$ -chitin) allows tight packaging of the chitin microfibrils and provides mechanical strength and stability to insect cuticle (Kramer and Koga, 1986; Merzendorfer, 2006).

### **Chitin synthesis**

In insects, synthesis of chitin is a continuous and crucial process required for growth and development. Chitin formation is catalyzed by membrane-bound chitin synthase enzymes (Merzendorfer, 2006; Merzendorfer and Zimoch, 2003) which were first isolated from yeast and filamentous fungi (Bulawa et al., 1986; Silverman et al., 1988; Yarden and Yanofsky, 1991). The first insect chitin synthase gene was isolated and sequenced by Tellam and colleagues from *Lucilia cuprina*, the Australian sheep blowfly (Tellam and Eisemann, 2000; Tellam et al., 2000). Recently, orthologs of this gene have been identified in the genomes of many other insect species such as *D. melanogaster*, *M. sexta*, *A. gambiae*, *A. aegypti* and *T. castaneum* (Arakane et al., 2005; Gagou et al., 2002; Ibrahim et al., 2000; Tellam et al., 2000; Zhu et al., 2002; Zimoch and Merzendorfer, 2002). Based on the sequence conservation and the presence of alternate exons in the two *Chs* genes found in all insect species, the two genes are named *chitin synthase-A* (*Chs-A*) and *chitin synthase-B* (*Chs-B*) (Arakane et al., 2005; Gagou et al., 2002; Zhu et al., 2002; Zimoch and Merzendorfer, 2002). *Chs-A* is required for cuticular chitin synthesis while *Chs-B* is expressed in the gut epithelium for peritrophic matrix chitin formation. Chitin synthesis is a complex process that involves hydrolysis of trehalose to form glucose in cytosol, followed by a series of reactions involving successively glucose-6-phosphate, fructose-6-phosphate, glucosamine-6-phosphate, *N*-acetylglucosamine-6-phosphate, *N*-acetylglucosamine-1-phosphate

and UDP-*N*-acetylglucosamine. Finally, integral membrane-bound chitin synthase-A enzyme with catalytic site facing cytoplasm utilizes UDP-*N*-acetylglucosamine (UDP-GlcNAc) as a substrate for chitin synthesis (Merzendorfer, 2005). The exact mechanism of secretion of chitin in the extracellular cuticle is still unknown. Based on electron microscopic analysis, chitin chains protrude from the tip of the microvilli on the apical side of the epithelial cells (Locke and Huie, 1979). Chitin synthase activity has been shown to require divalent cations such as Mg<sup>2+</sup> or Mn<sup>2+</sup> (Duran et al., 1975; Kramer and Koga, 1986). Whether or not a primer is needed to initiate the synthesis of chitin chains has not been resolved as yet.

### **Chitin organization in the cuticle**

Procuticular chitin synthesized by integral membrane bound protein chitin synthase-A (Chs-A) is deposited in the form of bundles, called microfibrils. Several such chitin microfibrils are arranged in parallel to form the chitin sheets, which are then stacked helicoidally along the apical-basal axis of the procuticle to make chitin laminae (Moussian, 2010; Vincent and Wegst, 2004). Adjacent chitin laminae from different sources of cuticle are oriented with respect to one another at a characteristic and constant angle to form helicoidal bundles known as Bouligand's structure (Moussian, 2010). Helicoidal arrangement of chitin gives cuticle flexibility while maintaining the ability to withstand stress. The association of chitin bundles and/or laminae with chitin may be responsible for the wide-ranging properties of cuticles in different parts of the same insect. For example, the beetle elytron is rigid while the underlying wing tissue is flexible. There are qualitative and quantitative differences in the composition of cuticular proteins associated with these tissues (Jasrapuria et al., 2010) (Dittmer, personal communication).

### **Proteins associated with chitin in the cuticle**

Different cuticular proteins have been identified and are thought to help in organization of procuticular chitin. Some representative cuticular proteins with Rebers and Riddiford [R&R] consensus (CPR family) and Tweedle family have been reported to bind to chitin *in vitro* (Willis, 2010). Two families of cuticular proteins have another chitin-binding motif called the peritrophin-A domains. This motif was found originally in a group of proteins called peritrophins that were extracted from the peritrophic matrix using strong chaotropic agents (Tellam et al., 1999). One of these families has three tandem peritrophin-A domains while the members of the other family have only one copy of this motif. The first group of cuticular proteins known as the

“obstructor family” or CPAP3 family (cuticular proteins analogous to peritrophins with 3 peritrophin A domains) may be important for organization of chitin. Consistent with this idea, mutations involving genes encoding many of these chitin-binding proteins or RNAi of these genes results in molting defects and lethality. (Barry et al., 1999; Behr and Hoch, 2005; Jasrapuria et al., 2010). The functions of the other family, CPAP1 with one peritrophin-A domain are still under investigation (Jasrapuria, Ph. D. Thesis, 2011, Kansas State University)

## **Molting in insects**

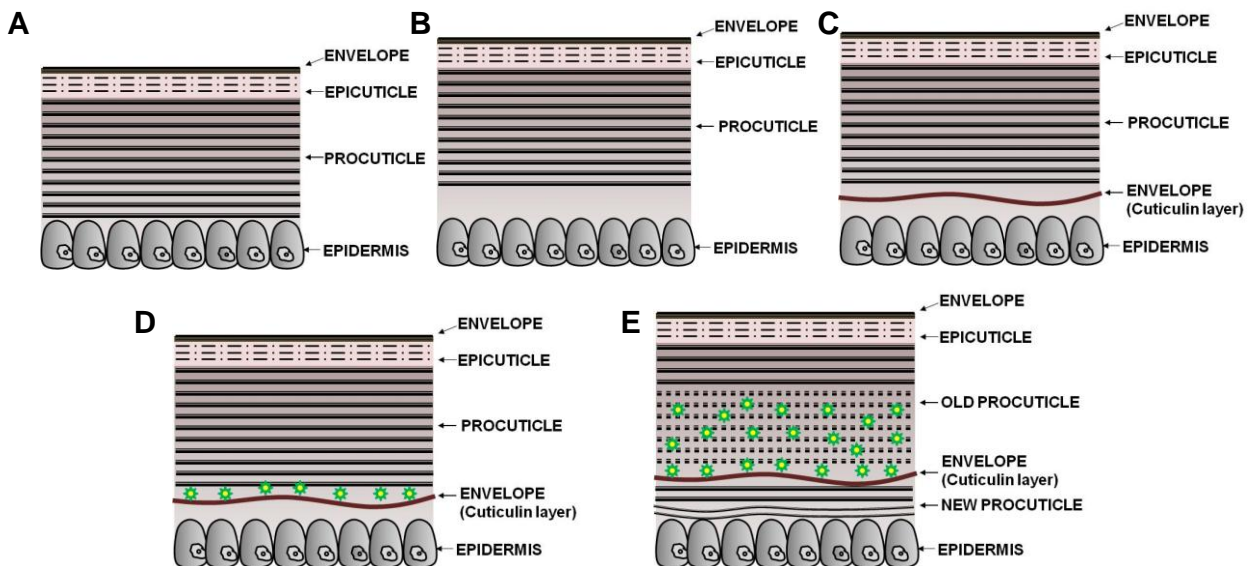
In addition to synthesizing and organizing cuticle during growth and development insects have to undergo a process of periodic molting, wherein old cuticle is replaced with a new one. Molting in insects is initiated by the hormone, ecdysteroid, followed by apolysis. The term apolysis was first introduced by Jenkin and Hinton in 1966 to describe the detachment of old cuticle from the underlying epithelial cells during the process of molting. This separation creates an apolytic space (Jenkin and Hinton, 1966) filled with molting fluid containing chitinolytic and proteolytic enzymes to degrade the old cuticle (Passonneau and Williams, 1953). Plasma membrane of epithelial cells and extracellular cuticle are in intimate contact during intermolt periods. To synthesize new layers of chitin during molting, old cuticle needs to be detached from the underlying epidermal cells (Passonneau and Williams, 1953). During the intermolt period of the larger canna leafroller, *Calpodes ethlius*, the apical plasma membrane of epithelial cells is not flat; rather it is folded into protruding structures called microvilli. The top surfaces of the numerous microvilli that come into contact with the innermost (bottom) layer of the cuticle are flat, and have been called plasma membrane plaques (Locke and Huie, 1979). The close association between these plaques and the endocuticle suggests that these could be the regions involved in cuticle synthesis. Electron microscopic analysis indicated the presence of large number of exocytotic vesicles just below the plaques, presumably containing chitin/protein cargo for cuticle synthesis at the surface of the plasma membrane of epithelial cells (Locke and Huie, 1979). Based on the above observations, it is likely that membrane bound chitin synthase enzyme locates at the apical plasma membrane plaques and synthesizes chitin microfibrils which are then transported into the extracellular cuticle by unknown mechanism. An extensive study of importance of plaques during molting and cuticle development was performed by Locke and Huie (1979). During the process of molting, ecdysial droplets start accumulating at the interface

of the plasma membrane and the overlying endocuticle causing detachment of the plaques from the epithelial cells. At this time microvilli and plasma membrane plaques of the epithelial cells start to regress. By the time ecdysial droplet synthesis is over, plaques completely disappear, leaving a smooth surfaced plasma membrane. Increase in ecdysone level is shown to be associated with the disappearance of the plaques. The period without plaques corresponds to the time of active RNA synthesis with multi-lobed nuclei and lots of ribosomes (Locke, 1970). Although cell division is concomitant with apolysis in some insects (Wigglesworth, 1973a; Wigglesworth, 1973b), mitosis may not be required in all insect species for initiation of apolysis (Prakash, 2008). Apolysis begins as plaques are lost, leading to separation of epithelial cells from the cuticle. Formation of microvilli is a very rapid process. As soon as the process of apolysis is over, plaques are redifferentiated to form the new cuticle. Study by Locke (1979) indicated that while all three different layers of cuticle are synthesized by the same plaques, the latter are replaced each time during molts to synthesize a new cuticle. It is interesting to notice that loss of plaques precedes detachment of the old cuticle from the epithelial cells. Once cuticle is detached from microvilli and underlying cytoskeleton, plaques have cuticular fibers associated with them, suggesting a role for plaques in adhesion of cuticle with the underlying epithelial cells (Locke and Huie, 1979). Electron microscopic analysis of *Podura aquatica* cuticle by Noble-Nesbitt (1963) has shown secretion of small smooth vesicles (also referred as foam formation) on the surface of apical plasma membrane of epithelial cells (Noble-Nesbitt, 1963). Foam formation is predicted to lift the old cuticle from underlying epithelial cells. From the observation from different cuticle it can be summarized that the loss of plaques on the apical plasma membrane precedes apolysis.

Molting fluid was first noted by Malpighi in 1669 as a transparent fluid required for molting (Passonneau and Williams, 1953). The apolytic space created between old cuticle and the underlying epidermal cells is filled with molting fluid to initiate the degradation of the old cuticle. Electron microscopic analysis of *Calpodes ethlius* molting larvae has shown accumulation of exuvial droplets at the surface of the epithelial cells after apolysis (Locke and Krishnan, 1973). These droplets were hypothesized to contain inactive enzymes which were thought to be activated after cuticulin layer formation is complete. The electron microscopic analysis of *Podura aquatica* cuticle at molt showed granules formation at the apical surface of epithelial cells after apolysis (Noble-Nesbitt, 1963). Granular bodies of about 100  $\mu$ m diameter

were predicted to be protein bearing entities. Noble-Nesbitt predicted that these granules release active enzymes in the apolytic space once the cuticulin layer synthesis is completed. Although the importance of molting fluid in degradation of old cuticle is found in old literature, its content and composition remained a mystery until 1950s. The presence of enzymes in the molting fluid was predicted from its ability to degrade old cuticle (Passonneau and Williams, 1953; Tower, 1906). In 1953, Passanneau and Williams described the presence of chitinases and proteases in the molting fluid of *Cecropia* silkworm. Their study indicated molting fluid as a colorless gel resembling egg albumin consistency having chitinase and protease activity (Passonneau and Williams, 1953). Chitinases in the molting fluid of *Manduca sexta* larvae were predicted to be in an inactive form (Bade and Shoukima, 1974). Further analysis in different insects has detected active chitinases and proteases in the molting fluid of different insects (Koga et al., 1992). The full length cDNA clone for *M. sexta* chitinases was isolated by Kramer et al. (Kramer et al., 1993). Since then, chitinase cDNAs have been cloned and isolated from different insect species such as *D. melanogaster*, *T. castaneum*, *A. gambiae* and *B. mori* for which genome is fully sequenced (Arakane and Muthukrishnan, 2010; Zhang et al., 2011a; Zhang et al., 2011b; Zhu et al., 2008b; Zhu et al., 2004). Domain organization of chitinases indicates the presence of an N-terminal signal peptide and single or multiple chitin binding and catalytic domains. As predicted, chitin binding assays in *T. castaneum* and *D. melanogaster* indicated tight binding of chitinases with chitin (Zhu et al., 2008a). The presence of a large family of chitinase genes in all insect species could indicate functional specialization and/or their tissue-specific requirements for chitin degradation. For example, in *T. castaneum* chitinase-5, chitinase-7 and chitinase-10 are cuticle specific chitinases and several others exhibit gut-specific expression patterns (Zhu et al., 2008c). Ubiquitous expression of epidermis-specific chitinases in *T. castaneum* indicated their requirement in all stages of cuticle development, while some other chitinases (chitinases 2, 4, 6 and 8 for example) were expressed in the midgut tissue only in the active feeding larval stages, indicating their role in the gut for peritrophic matrix degradation and/or digestion of chitinous material in the diet (Zhu et al., 2008c). Down-regulation of cuticle-specific chitinase expression has been shown to affect molting in *T. castaneum* (Zhu et al., 2008c). Although chitinase expression is detected in different stages of development, its localization has not been studied in detail. A single study on immunostaining of chitinases in spruce budworm molting larvae indicated localization of chitinases in the molting fluid, apolyzed old cuticle and in the

“Schmidt’s layer” (the site of new chitin synthesis) (Zheng et al., 2003). It was intriguing that these workers observed substantial chitinase staining in the Schmidt’s layer, which is right above the surface of the epithelial cells. From this study it was not clear whether chitinases were present on the apical side of the epithelial cells or were released into the extracellular space where chitin was being synthesized. From the available data and the absence of membrane-spanning/anchoring regions in the deduced amino acid sequences of chitinases we can predict that the chitinase 5 and chitinase 10 synthesized by epithelial cells would be secreted into the newly synthesized procuticle. Several mechanisms can be predicted to explain how newly synthesized chitin could be protected from degradation by chitinases. First, chitinases that co-localize with chitin could very well be in the inactive state and activated subsequently by limited proteolysis. Second, chitinases could be inhibited by some other proteins in the newly synthesized cuticle. Finally, chitin could be masked with some other chitin binding proteins, thereby avoiding direct interaction with chitinases.



**Figure 1.3 Classical model of molting in insects.**

Molting in insects starts with separation of old cuticle from the epithelial cells (B=apolysis). Apolytic space is then filled with molting fluid containing chitinolytic and proteolytic enzymes. A thin envelope (also known as cuticulin or outer epicuticle layer) is formed to avoid direct contact between chitinases (green shapes) from molting fluid and the site of new chitin synthesis (C). At the same time when old cuticle is being degraded by chitinases (D), new cuticle is formed and arranged in a laminar form (E).

We reasoned that the localization of chitinases with respect to newly developing chitin in the cuticle in more detail will provide some more insights into the mechanism of protection of chitin in the procuticle. In addition to chitinolytic enzymes, molting fluid contains proteases that degrade the cuticular proteins bound with chitin and makes chitin more accessible for chitinase action. Protein-free chitin can then be attacked by exo- and endo- chitinases and be degraded into monomers (Fig 1.3).

## **Cuticulin layer**

Molting fluid contains chitinolytic and proteolytic enzymes that degrade the old cuticle during molting. In order to protect the newly-forming chitin, a thin “cuticulin layer” or “an envelope” is formed. Envelope layer has been predicted to act as a physical barrier that prevents direct contact between molting fluid chitinases and the chitin in the newly synthesized cuticle. Towards the end of the period of ecdysial fluid formation, microvilli and plaques disappear and reappear on the apical side of the epithelial cells during the following instar for the synthesis of the new cuticle. The envelope is the first layer of the new cuticle synthesized during molting. This waxy envelope layer has been shown to be resistant to enzymatic actions (Wigglesworth, 1947b; Wigglesworth, 1948) and therefore predicted to protect the newly synthesizing cuticle and epidermal cells underneath from coming into contact with chitinases and proteases present in the molting fluid. A thin dense layer of envelope covering the whole epithelial cell surface is initially formed as disconnected smaller fragments that later fuse to form the intact and continuous envelope layer (Moussian et al., 2006). The gaps during early envelope synthesis have been proposed to provide space for ecdysial droplets to move from the epithelial cells to the apolytic space (Noble-Nesbitt, 1963). Once completely synthesized, the envelope covers the entire surface of the array of epithelial cells. The flow of enzymes has been predicted to be forward from epithelial cells to the apolytic space and not in the opposite direction to the basal side. Ecdysial droplets accumulate outside the completely formed envelope and are believed to contain inactive molting enzymes for old cuticle degradation (Passonneau and Williams, 1953). Protection of newly synthesized cuticle from degradation by molting enzymes has been a mystery. Different theories have been proposed and debated in past to account for the stability of the new cuticle. First, it is believed that molting fluid secreted in the apolytic space is protected from coming into contact with the underlying epithelial cells by the envelope. Second, newly synthesized layers of chitin are



predicted to be devoid of any chitinases and proteases. Finally, activation of molting enzymes has been predicted to occur at a very late stage in molting (Passonneau and Williams, 1953) when, newly synthesized cuticle is believed to be almost completely synthesized and tanned by creating protein-chitin complex. Tanning of newly synthesized procuticle is predicted to be enough for protecting chitin from molting fluid chitinases.

However, the above rationale is mostly hypothetical not supported by any experimental evidence. There is no clear-cut evidence for the presence of inactive forms of chitinases in the molting fluid. Multiple forms of chitinases differing in molecular weights have been detected in molting fluid, but all of them have enzymatic activity (Koga et al., 1992). Further, recombinantly expressed *M. sexta* chitinase, which is secreted into the medium after the cleavage of the signal peptide, is enzymatically active (Gopalakrishnan et al., 1995). Secondly immunolocalization of chitinase in the assembly zone of the newly forming cuticle reported by Zheng et al., (2003) clearly contradicts the hypothesis that the envelope provides a physical barrier for chitinases from reaching the procuticle. Thirdly, the basic tenet of the above hypothesis has not been tested by determining the relative locations of chitin and chitinases within the cuticle during molting. The current study remedies this deficiency by localizing both chitin and chitinases in different cuticles using high resolution confocal microscopy. The major finding of my research is that newly synthesized chitin and chitinase are indeed colocalized within the procuticle disproving the hypothesis that the new envelope is a physical barrier between molting fluid chitinases and chitin in the new cuticle. In an attempt to find an alternate explanation for the stability of chitin in the new cuticle at a time when the chitin in the old cuticle is degraded, I have examined the role of two other proteins that have been implicated in maintenance of cuticle integrity. One of these proteins, Knickkopf, does bind to chitin and is intimately associated with chitin in the procuticle. The results presented in this dissertation demonstrate that Knickkopf is essential for protecting chitin from being degraded by active chitinases that are secreted at the time of molting. Second protein, Retroactive, influences this process by helping target Knickkopf to its extracellular location.

## Outline of this thesis

With the broad goal of defining basic mechanisms of organization and turnover of chitin during molting in insects, the relative locations of chitin and chitinases was examined using the elytral and body wall cuticles of the red flour beetle, *Tribolium castaneum*. As presented in the literature review (**Chapter 1**), there is ample evidence to suggest the presence of enzymatically active chitinases during molting. However, the mechanism of protection of newly synthesized chitin from these chitinases is still unknown. Accordingly, **Chapter 2** provides experimental data on localization of chitin, chitinases as well as another chitin-binding protein Knickkopf in the developing new cuticle of *T. castaneum* pharate adult stage. This chapter also explores the molecular mechanism of protection of newly synthesized chitin from chitinases by the Knickkopf protein. In **Chapter 3**, the importance of protein Retroactive in proper trafficking of Knickkopf from epithelial cells to within the developing new procuticle is described. In **Chapter 4**, the roles of both Knickkopf and Retroactive in serosal cuticle deposition during embryonic development of *T. castaneum* is explored particularly focusing on how they affect chitin levels and its laminar organization. In **Chapter 5**, I report the presence of genes encoding two more Knickkopf-like proteins in *T. castaneum* genome and their role in molting and cuticle organization. Finally, in **Chapter 6**, I summarize the major findings of this thesis and experiments for further study in the future.

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## **Chapter 2 - The Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton**

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## **Abstract**

During each molting cycle of insect development, synthesis of new cuticle occurs concurrently with the partial degradation of the overlying old exoskeleton. Protection of the newly synthesized cuticle from molting fluid enzymes has long been attributed to the presence of an impermeable envelope layer that was thought to serve as a physical barrier, preventing molting fluid enzymes from accessing the new cuticle and thereby ensuring selective degradation of only the old one. In this study we show using the red flour beetle, *Tribolium castaneum*, as a model insect species that an entirely different and unexpected mechanism accounts for the selective action of chitinases and possibly other molting enzymes. The molting fluid enzyme chitinase, which degrades the matrix polysaccharide chitin, is not excluded from the newly synthesized cuticle as previously assumed. Instead, the new cuticle is protected from chitinase action by the protein Knickkopf (TcKnk). TcKnk co-localizes with chitin in the new cuticle and organizes it into laminae. Down-regulation of *TcKnk* results in chitinase-dependent loss of chitin, severe molting defects and lethality at all developmental stages. The conservation of Knk across insect, crustacean and nematode taxa suggests that its critical roles in the laminar ordering and protection of exoskeletal chitin may be common to all chitinous invertebrates.

## Introduction

During development, insects must undergo periodic molting to accommodate growth and to overcome the rigid constraints imposed by portions of their chitinous exoskeletons (Chang, 1993; Reynolds and Samuels, 1996). This process entails the complete replacement of the entire outer shell of the insect, including digestion, resorption and recycling of the innermost pliable layers (layers closer to the epithelial cells), and shedding of the outer (superficial), more highly sclerotized and waterproofed layers, which are either discarded or in some cases ingested for further recycling (Chang, 1993; Locke, 2001; Merzendorfer and Zimoch, 2003; Reynolds and Samuels, 1996). The molting process is hormonally initiated by 20-hydroxyecdysone and begins with the epidermis secreting what will become the outer layers of the new cuticle, which separate the epidermal layer from the overlying old cuticle. An “apolytic space” then forms, separating new (inner) from old (outer) cuticles (Locke and Huie, 1979). With the delicate epidermis now protected by the first layers of new cuticle, the molting fluid in the apolytic space can digest the inner layers of the outer (old) cuticle. According to long-held dogma, protection of the new cuticle from degradation by molting fluid enzymes is conferred by a thin, non-chitinous envelope (previously termed the “cuticulin” layer or the “outer epicuticle”) deposited by the epidermal cells just prior to the secretion of new cuticular chitin underneath (Locke, 2001). This envelope was believed to form a protective barrier against proteolytic and chitinolytic enzymes of the molting fluid, thereby confining their actions to the proximal layers of the old (outer) exoskeleton while preventing digestion of the newly deposited layers of the replacement cuticle (Locke, 2001; Reynolds and Samuels, 1996).

However, the role of the envelope in preventing inward diffusion of molting enzymes has never been convincingly demonstrated, and molecular determinants that ensure selective degradation of old cuticle while protecting the new cuticle are still a matter of speculation. A finding that chitinolytic or proteolytic enzymes in molting fluid are excluded from the nascent procuticle would lend some support to the view that the envelope forms a barrier for protection of newly secreted layers of chitin or protein. Zheng et al. (Zheng et al., 2003) immunolocalized a chitinase in spruce budworm (orthologous to chitinase-5 of *Tribolium castaneum*) (Zhu et al., 2008a; Zhu et al., 2008b) in the molting fluid and the integument but did not determine whether this chitinase was colocalized with chitin in the newly synthesized cuticle. In this study we refute the venerable notion that access of molting enzymes to nascent cuticle is restricted by the

presence of a protective envelope, and provide evidence for an entirely unexpected alternative mechanism. We show that the insensitivity of new cuticle to molting enzymes, specifically chitinase, is conferred by the protective effects of the protein Knickkopf (Knk), and that Knk has an additional essential role in the proper organization and layering of the exoskeletal laminae.

## **Materials and Methods**

### ***Tribolium* strains**

The *Tribolium castaneum* GA-1 strain was used for all experiments. Insects were reared in whole wheat flour containing 5% brewer's yeast at 30°C under standard conditions as described previously (Beeman and Stuart, 1990). *Tribolium* life cycle is divided into four distinct stages of development named embryonic (4-5 days), larval (~15 days), pupal (5 days) and adult stage (2 months to 2 years). Most of this study was done using penultimate instar (young) larvae, last instar (late or mature) larvae and pharate adults (one day before adult eclosion).

### **Identification, cloning and phylogenetic analysis of *TcKnk***

The *D. melanogaster* Knk protein sequence was used as the query to detect putative homologs in the *T. castaneum* genome database by using the tblastn program. This resulted in the identification of a presumptive orthologous gene, which we have named TcKnk (JN314843.1). TcKnk-specific primers (forward primer 5'-GCGGTTCTCGTGTAAGTATGTG-3' and the reverse primer 5'-TCTCAGATGTTTATGGCCTCTCTG-3') were used to amplify the 2,013 bp-long complete coding sequence of TcKnk using pupal stage cDNA as template. The amplification product was subsequently cloned into pGEM-T vector (Promega) and its identity was confirmed by DNA sequencing. Sequence alignment of the predicted Knk proteins from different insects, other arthropod, as well as nematode species was carried out using the ClustalW software prior to phylogenetic analysis. MEGA 4.0 (Tamura et al., 2007) was utilized to construct the consensus phylogenetic tree using the neighbor joining method.

### **Determination of expression profile of TcKnk**

Using the RNeasy Mini kit (Qiagen), total RNA was prepared from pools of embryos, young larvae, last instar larvae, pharate pupae, pupae, young adults (0-1 h after adult eclosion) and

mature adults (10 d-old). For determining tissue specificity of expression, RNA was extracted from midgut, hindgut and carcass (whole body without gut) of the last instar feeding stage larvae. cDNAs were synthesized from total RNA using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) as described previously (Arakane et al., 2005a). RT-PCR was done to check the expression profile using the gene-specific primer pair: forward 5'-AAAGATTTCCAAGTGTCGTGG-3' and reverse 5'-CCAAATAGGCTTTAAAAGTCCG-3'. cDNA amplification product for *T. castaneum* ribosomal protein S6 (RpS6) was used as an internal loading control for RT-PCR (Arakane et al., 2011).

### dsRNA synthesis

dsRNAs were prepared as described earlier (Arakane et al., 2005a). Two unique regions within TcKnk were chosen as templates for the synthesis of gene-specific dsRNAs (Table S1). The dsRNAs were synthesized from linearized templates by using the AmpliScribe T7-Flash transcription kit (Epicentre Technologies).

### Injection of dsRNA into *T. castaneum*

dsRNAs were injected into young larvae, last instar larvae and pharate pupae by using a microinjection system and a dissection stereomicroscope as described earlier (Tomoyasu and Denell, 2004).

**Table 2.1 Primers used for dsRNA synthesis.**

dsRNA		Sequence (5'-3')	Nucleotide positions	Length
1	F	TAATACGACTCACTATAGGGTGGCCAAACATCCACG	1449-1900	451
	R	TAATACGACTCACTATAGGGTCCATGTTTGACTGCG		
2	F	TAATACGACTCACTATAGGGTTGGGACAATATAAAGGTG	1036-1201	165
	R	TAATACGACTCACTATAGGGTAAATTGAGCCCTCGG		

### **Measurement of transcripts for *TcKnk* after dsRNA treatment**

Total RNA was prepared from pools of three insects after injection of dsRNA by using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was carried out to determine the effect of dsRNA injections on level of transcripts by using gene-specific primers. A pair of *T. castaneum* ribosomal protein-S6 (RpS6) gene primers was used as an internal control to monitor equal loading of cDNA for analysis of transcript levels.

### **Expression of TcKnk**

A full-length cDNA clone of TcKnk was used as a template to amplify a sequence corresponding to the complete coding region of the TcKnk protein. The primer pair containing appropriate restriction enzyme sites (forward primer 5'GAGACCCGGGATGTTACTACTCTTCGTT 3' and a reverse primer 5' GAGATCTAGATTACACGAATTGCTTAAC 3') was used to facilitate directional cloning of the TcKnk open reading frame (ORF) in the pVL1393 expression vector (BD Pharmingen). PCR-amplified TcKnk ORF DNA and the pVL1393 vector DNA were digested with the same pair of restriction enzymes and ligated as described previously (Zhu et al., 2008a). Recombinant baculovirus for expression of TcKnk protein was constructed using BaculoGold DNA and 1 µg of the recombinant baculovirus transfer vector pVL1393 DNA containing the ORF for the TcKnk protein and amplified to obtain a high titer virus. Hi-5 cells (*Trichoplusia ni* cell line) were used to express the TcKnk protein as described earlier (Zhu et al., 2008a).

### **PI-Phospholipase-C (PLC) treatment and western blot analysis**

Hi-5 cells were infected with a recombinant baculovirus containing the TcKnk open reading frame and incubated at 30°C for 3 d for expression of TcKnk protein. Three days post-infection, media (1 ml) and Hi-5 cells were harvested from each well as a control to check the levels of TcKnk protein at the beginning of the experiment. Subsequently, 1 ml of fresh medium containing 100 µl of phosphatidylinositol-specific phospholipase-C (PI-PLC) from *Bacillus cereus* (7.89 units/mg, Sigma Aldrich) or medium alone (mock) was added. PI-PLC- or mock-treated cells were incubated at 30°C for 4 h followed by collection of the media and cells. Cells were then resuspended in 1 ml of PBS, lysed in sample buffer, and aliquots of the supernatant were analyzed by SDS-gel electrophoresis on 4-12% gradient SDS gel (Invitrogen). TcKnk

released as a result of PI-PLC treatment was detected by western blot using the anti-Knk antibody.

### **Immunohistochemistry and confocal analysis**

dsRNA-treated pharate pupae were collected at the pharate adult stage (pupa day 5, n=5) to analyze multiple cuticles (old pupal cuticle, new adult elytral as well as body wall cuticle) and were fixed in 4% para-formaldehyde at 4°C overnight followed by treatments with a series of solutions with increasing sucrose concentrations (12, 15, 18 and 20% sucrose). Cryosections (20 µm thick) of the fixed pharate adult elytral and body wall samples were stained for TcKnk and TcChs-A proteins using *D. melanogaster* Knk rabbit antiserum (1:100) and *T. castaneum* Chs-A rabbit antiserum (1:50) as primary antibodies, respectively. Alexa 488 goat-anti-rabbit IgG (1:1000 dilution, Ex/Em=495nm/519nm) was used as secondary antibody for green fluorescence detection of the respective proteins. Rhodamine-conjugated chitin-binding probe (1:100 dilution, New England Biolabs, Ex/Em=544nm/572nm) and DAPI (1:15, Ex/Em=350nm/470nm) were used for the chitin and nuclei staining, respectively. The blue spheres in all images indicate nuclei. Confocal microscopy was performed on an LSM META 510 laser scanning confocal microscope equipped with lasers capable of 405 nm (DAPI), 488 nm (Alexa 488) and 543 nm (Rhodamine-conjugated chitin-binding probe) excitation. Following filters were used to take images, DAPI= BP= 420-480 nm, Alexa 488= BP= 505-530 nm and Rhodamine conjugated chitin binding probe = LP= 585 nm. An oil objective (40 X/1.3 NA) with 8 X zoom was used for the present study. Sample autofluorescence was checked at all wavelengths without the addition of the antibody (Appendix B). 1024x1024 pixel, 8 bit images were collected as multiple tracks. Single optical slices roughly at the middle of the cryosection were taken. Images were saved as tiff files after adding scale bars in ImageJ software. Each image was then redefined to 800dpi using windows 7-Adobe Photoshop CS3 and put in an 8cm x 6cm size frame.

### **Chitin content analysis**

dsRNA-treated pharate pupal and pharate adult insects were collected for chitin content analysis. Chitin content was measured using a modified Morgan-Elson method as described previously (Arakane et al., 2005b). Insects treated with dsRNA for TcVer and TcChs-A were used as negative and positive experimental controls, respectively (n =5).



### **Colloidal chitin-binding assay**

For colloidal chitin binding assay, TcKnk expressed in Hi5 cells was extracted using Mem-Per kit as per the manufacturer's instructions (Pierce) and referred to as TcKnk with GPI anchor (GPI+). TcKnk without GPI anchor was extracted from Hi5 cells expressing TcKnk using PI-PLC (Sigma). Colloidal chitin prepared from crab shell chitin was used for the binding assay at a final concentration of 1  $\mu\text{g}/\mu\text{l}$  (Zhu et al., 2008a). The colloidal chitin-binding assay was carried out as described previously (Zhu et al., 2008a) with the following modifications. Colloidal chitin (50  $\mu\text{l}$ ) was mixed with membrane protein extracts containing ~100 ng of protein. This mixture was incubated at room temperature for 1 h with end-to-end rotation and then centrifuged for 10 min at 4°C. The pellet was then washed with 10 mM sodium phosphate buffer (pH 8.0) followed by centrifugation for 4 min. The supernatant was saved as a flow-through fraction for loading on the gel. The relative affinity of TcKnk for the chitin pellet was further tested by two additional treatments of 10 mM sodium phosphate buffer containing 1 M NaCl (pH 8.0), followed by elution with a solution containing 1% Calcofluor which is known to elute some chitin-binding proteins. Finally, the pellet was re-suspended in 50  $\mu\text{l}$  of SDS gel loading buffer and boiled for 10 min. The presence of TcKnk in each fraction was detected by western blot analysis using the anti-Knk polyclonal antibody and the horseradish peroxidase-conjugated secondary antibody detection system (Bio-Rad).

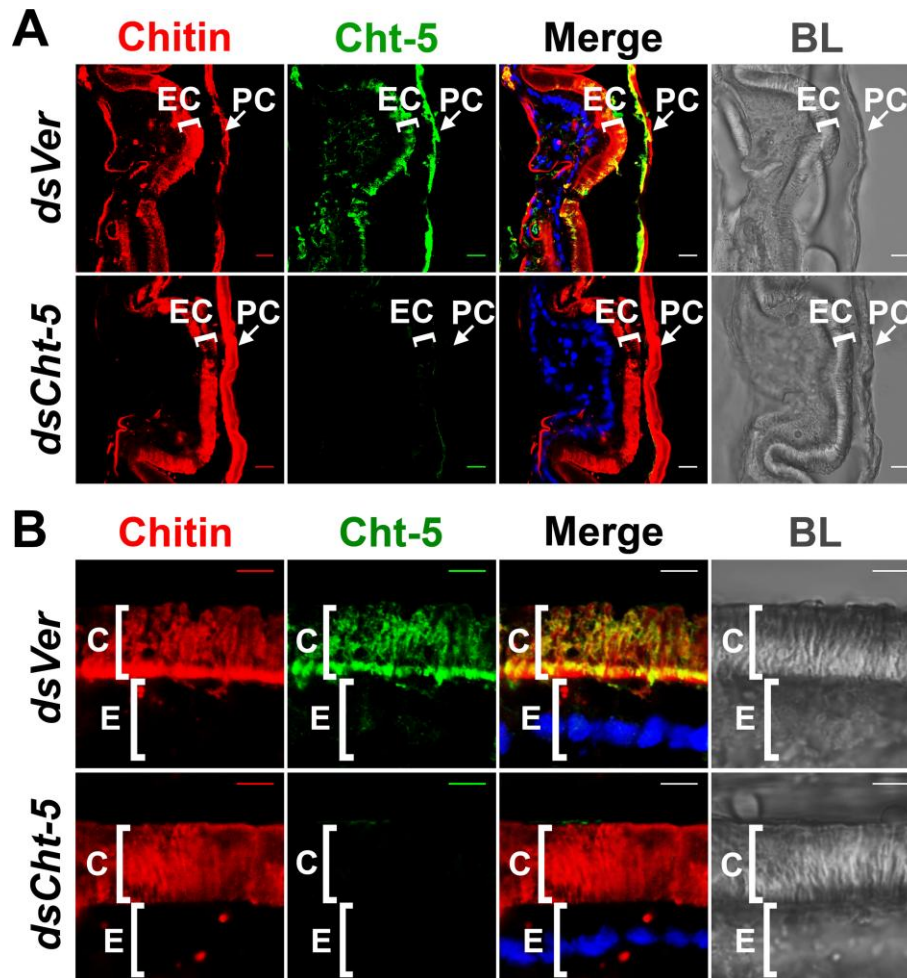
### **Transmission electron microscopy**

dsRNA-treated insects were collected at the pharate adult (n=3, multiple sections) stage of development and fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2 - 7.4) for 24 h at room temperature (RT) with constant rotation. Samples were then washed 3 times for 5 min each in 0.1 M sodium cacodylate buffer at RT with constant rotation followed by post-fixation with 1-2% osmium tetroxide in 0.1 M sodium cacodylate buffer at RT with constant rotation for 1-2 h. Samples were then washed with 0.1 M sodium cacodylate buffer at RT 3 times for 5 min each with constant rotation and stained with 2% uranyl acetate for 1 h. Samples were rinsed with 0.1 M sodium cacodylate buffer, dehydrated using an ascending acetone gradient (50, 60, 70, 80, 90, 95 and 100%), infiltrated with EMBED 812/Araldite resin (1:1 propylene oxide: resin mixture for 10 min followed by a 1:2 propylene oxide: resin mixture for 20 min and 100% resin for 16 h), vacuum infiltrated for 1

h, embedded in flat molds and the resin was cured in a drying oven at 60°C for 24-48 h. Samples were then trimmed, thin-sectioned (silver to gold section), absorbed on to 200 mesh copper grids and imaged on a CM-100 TEM (FEI Co.).

## Results and Discussion

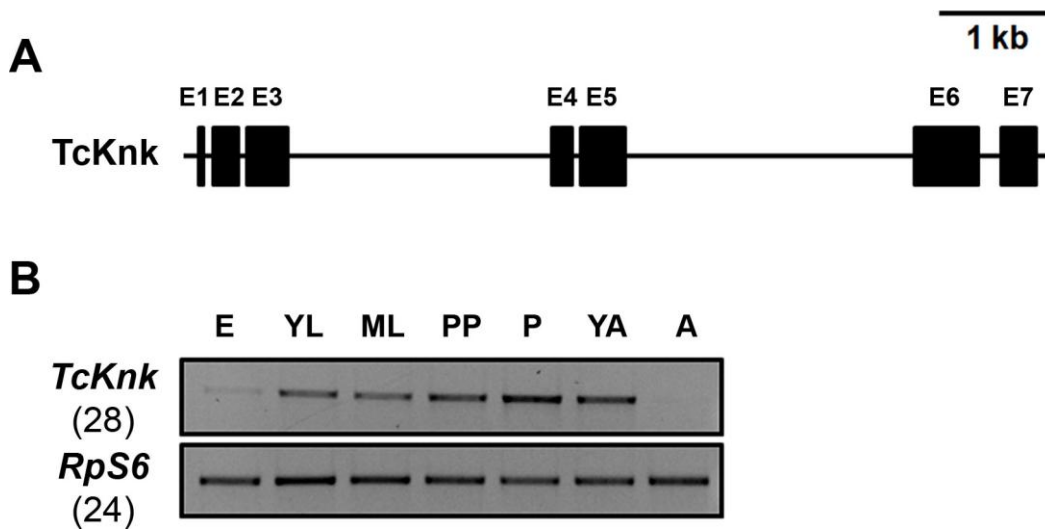
### Co-localization of chitinase with chitin in the newly synthesized cuticle.



**Figure 2.1 Chitinase co-localizes with chitin in the newly-synthesized cuticle.**

Cuticular chitin (red) in (A) elytra and (B) lateral abdominal body walls of pharate adults subjected to RNAi for *TcVermilion* (*dsVer*) and *TcChitinase-5* (*dsCht-5*) were detected using a fluorescent rhodamine-chitin-binding protein probe. *TcCht-5* (Cht-5-green) was immunostained with polyclonal antibodies raised against *M. sexta* group I chitinase. Bright-field images (BL) were used as a guide to delineate cellular and cuticular regions. EC, new adult elytral cuticle; PC, old pupal cuticle; C, cuticle; E, epithelial cells. Scale bar = (A) 10  $\mu$ m, (B) 5  $\mu$ m.

To establish the spatial relation between chitin in the newly synthesized cuticle and molting fluid chitinases, we simultaneously localized both of them within several cuticles of the red flour beetle, *Tribolium castaneum*, prior to the adult molt. The results revealed that chitinase is associated not only with the old (pupal) cuticular chitin but also with chitin in the newly formed pharate adult elytral cuticle (Fig 2.1A). A closer examination of the newly formed abdominal body wall cuticle (Fig 2.1B) showed that chitinase actually permeates the site of new chitin accumulation just outside the epidermal cell layer and throughout the entire procuticle. This unexpected discovery challenges the long-held view of an impermeable envelope that surrounds the new cuticle, limiting accessibility and protecting it from molting fluid enzymes.



**Figure 2.2 Exon–intron organization and developmental stage-specific expression pattern of *TcKnk*.**

(A) Schematic diagram of the exon–intron organization of *TcKnk* gene. The exon–intron organization of *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 *DmKnk*. Black solid boxes indicate exons and lines indicate the introns. (B) Expression of *TcKnk* during insect development. Total RNA was prepared from: E, eggs; YL, young larvae; ML; mature last instar larvae; PP, pharate pupae; P, pupae; YA, young adult and Y, adult stages. cDNAs synthesized from total RNAs using 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).

### **Effect of *TcKnk* on molting and chitin content.**

Deletion mutants of the gene, *knickkopf* (*knk*), exhibit cuticular defects and embryonic lethality in *Drosophila melanogaster* (Ostrowski et al., 2002). *Knk* has subsequently been found to be essential for the normal laminar organization (multiple layers of the cuticular chitin arranged parallel to the epithelial cells) of endocuticle at embryonic stages of growth (Moussian et al., 2006). However, the possible contributions of *Knk* to post-embryonic cuticle morphogenesis were unknown. To investigate these possible aspects of *Knk* function, we identified its ortholog in *T. castaneum* and named the gene *TcKnk* (Fig 2.2A). The encoded protein has a domain organization similar to that of *D. melanogaster* *Knk* (Moussian, 2010; Moussian et al., 2006) (Fig 2.3 and Fig 2.4A) and its mRNA is expressed throughout all post-embryonic stages of development up to the young adult stage, when cuticle deposition is essentially complete (Fig 2.2B). Transcripts of *TcKnk* were observed only in cells of epidermal origin, namely the hindgut and carcass (whole body minus gut), but were notably absent in the endodermally derived midgut, suggestive of a specialized role for *TcKnk* in cuticle formation (Fig 2.4B). As demonstrated previously with *D. melanogaster* *Knk*, recombinant full-length *TcKnk* protein expressed in an insect cell line can be released into the culture medium by treatment with a phosphoinositide-specific phospholipase-C (PI-PLC), indicating that it is a GPI-anchored membrane protein (Fig 2.5).

Consistent with a putative function in post-embryonic cuticle morphogenesis, dsRNA-mediated knockdown of *TcKnk* transcripts led to lethal molting defects at all stages of *Tribolium* development (larval-larval, larval-pupal and pupal-adult molts) (Fig 2.4C and Fig 2.4E). Molting defects in *TcKnk* dsRNA-treated beetles closely resembled those previously observed after dsRNA-mediated knockdown of *chitin synthase-A* (*TcChs-A*), which is essential for cuticular chitin synthesis (Arakane et al., 2005b). Quantitative chemical analysis revealed that insects subjected to RNA interference (RNAi) for *TcKnk* were severely deficient in total chitin, comparable to insects in which chitin synthesis was prevented by RNAi for *TcChs-A* (Fig 2.4D). Examination of cryosections after RNAi for *TcKnk* confirmed a near-complete loss of cuticular chitin in the body wall similar to that observed following *TcChs-A* knockdown (Fig 2.6A-B, column 1). These data reveal a crucial role of *TcKnk* in the deposition or maintenance of chitin in the newly formed cuticle.

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          *      20      *      40      *      60      *
DmKnk : MALSSFVLCRLQHLLIVILTIFLLCPAASAQNEEEDGPYRGKYLGKLNSYHHQVSGDVYAVNEYTFLI
TcKnk : -----MLLLFVLLLASSGSCQDSE---GPYRGKHIGKLNSYHHEVGGDVYAVDDYTLL

          80      *      100      *      120      *      140
DmKnk : VGFNYDGNGADTFFWSGASNRPGPQGFIVPDEYGKTNILDRYHNKDFTLTLPDRKKITEIKWLAVYDLSS
TcKnk : TSFSYDGNGADTFFWAGASNRPGPQGFIVPDEYGKTNVLARYLQKDFTLTLPDNKKITDIKWFAIYDLLS

          *      160      *      180      *      200      *
DmKnk : QNNFGDVYIPEEFDPMSQLGGTFSKRSHNVSSSSVEILDSKTIRIKDFTYDGRGKRTFFWTGVGPQPSS
TcKnk : QNTFGDYIIPEEFEPPTVQKIPQLASKSHSVSSGAVEIIDAKRIRLNDFRYNGQAKRAHFVVGVGAQFVS

          220      *      240      *      260      *      280
DmKnk : RGSKLPDERGYLDPIRQYNKETIELELPGDKTIFDIDWISVYDVADNENYGHVLFNDKLNVPPSIVKVTP
TcKnk : KGHKVPDENGYLDPLRAYKGETITLELPGDLTIFNIAWFSVFDLETNENLGSIVIPDGLNVPPSIVKVIP

          *      300      *      320      *      340      *
DmKnk : FEFSLPNCRQLHKDMCVSWEVFGPOITFQLSGVGNDYMSFGISGSDVSSOMIGSDVVVAIDDIRGYT
TcKnk : HEHHLPNCLQLHKDFCVSWDIFGPOITIQMAGQVDENSYMAFGMSGSKEQSQMLGSDVTVAYLDGYRGYA

          360      *      380      *      400      *      420
DmKnk : VDYNITSLAPCVQVLGQNKGVCRDDVVGGLDSFQLNTYSRKDGINTISFRRTLKSSDDGDKEIFLDRSNY
TcKnk : TDYNITALTPCVKVLGQYKGVCKDEIVGGQDSNQMHTATRENGINIITFRRALISPDPGDQEYPTEGSIY

          *      440      *      460      *      480      *
DmKnk : VIWAFGPLDSNEPAFHTYYPKSDIVIDFNTTEFVNDCFAFTKRAETTNPFWERTRITDATVRTFNAYL
TcKnk : IIWAMGRLDHNKEPTFHDIYPKTNISIELQPKEPSSNCFSFTR-SDTQLREPVWKGQIYDKTIRTFKAYL

          500      *      520      *      540      *      560
DmKnk : GPSGGLRGYQGLTNHVSSGLAWYINGYMIPELYLKRGLTYTFKVRGGNNPHSPEHYHPIVITDDPQGGYD
TcKnk : GPSGGRRGYQGTTGTSTTLAWYVNGLLAPELWLRRGLTYAFRVYGGNNPHSAEFYHPLIITDEPHGGFD

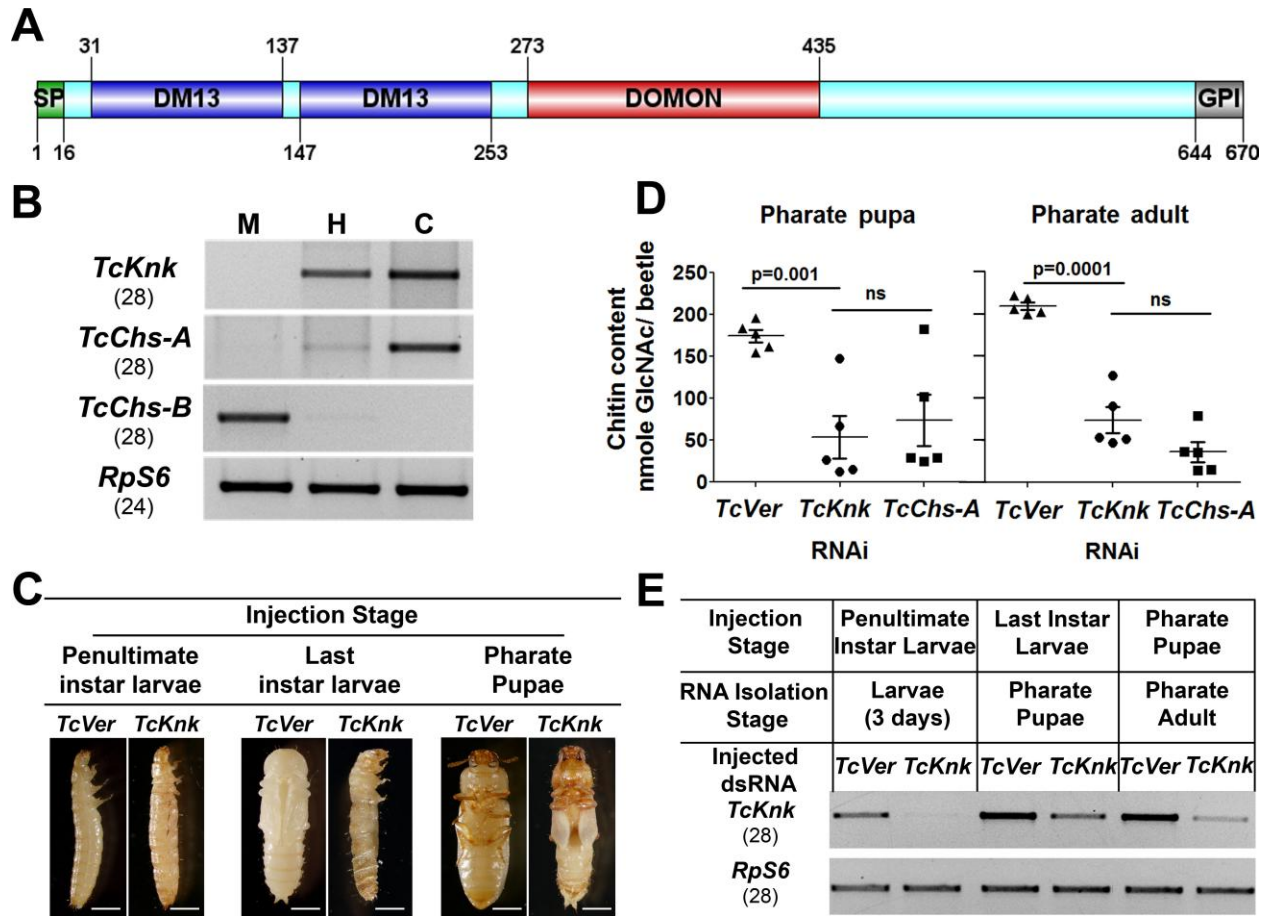
          *      580      *      600      *      620      *
DmKnk : RLSDAKQSEIRVLAGVEFTRRRGRPKTAAGPLCLSRYPQNSDRRLDNFPTFKKFNRSLITECVEGEPAL
TcKnk : RLSDEAQAKIRVLAGVEYSRRRGRPRPTAAGPLCLAKHQDSNDRRLDGFPTFKKFNRSIVSCEEGEPGV

          640      *      660      *      680      *
DmKnk : LEITPNITWPDTVYYNSFTHGNMGWKIHIVDSYTNLKSGSMGLSWSLCIILLPWIVLQN-
TcKnk : LEFTPNSSWPDIVYYNSFTQSNMGWKIHIDSFSSRTNGISKMCGNIFVIVSILFVKQFV

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**Figure 2.3 Amino acid sequence alignment of TcKnk and DmKnk.**

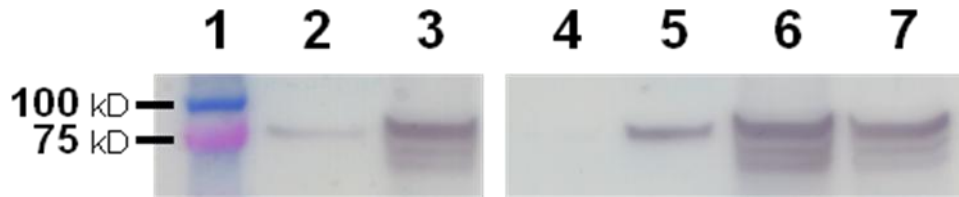
Shading of amino acid residues in gray color indicates identity. Underlines with different colors indicate different domains. Green, leader peptide; Blue- DM13 domains; Red, DOMON domain; Black underline, GPI-anchor specifying sequence.



**Figure 2.4 Domain organization, expression and role of *TcKnk* in maintenance of cuticular chitin.**

(A) *TcKnk* is composed of an N-terminal signal peptide (SP), two DM13 domains, a dopamine monooxygenase N-terminal like domain (DOMON) and a C-terminal GPI anchor. Domains were annotated using the SMART database and graphed using the protein domain illustrator program, DOG 1.0.3. (B) Tissue-specific expression pattern of *TcKnk* in the last instar larval midgut (M), hindgut (H) and carcass (C) of *T. castaneum*. *TcChs-A* and *TcChs-B* transcripts were used as carcass-specific and midgut-specific markers, respectively, to assess the purity of the tissue preparations, while *RpS6* was an internal loading control used to normalize for the differences in the concentrations of the cDNA templates. Numbers in parentheses indicate the cycle number for each of transcripts shown. (C) RNAi was carried out by injecting ~200 ng of *TcKnk* dsRNA into penultimate instar larvae, last instar larvae or pharate pupae. Representative images of terminal phenotypes are displayed along with controls injected with dsRNA for *Vermilion* gene (*TcVer*) (n = 20 each). Scale bar= 1 mm. (D) Quantitative analysis of chitin content from whole animals at pharate pupal and pharate adult stages was carried out using a modified Morgan-Elson assay and reported as mean  $\pm$  SE (n = 5). Statistical significance was computed by the Student's *t*-test. ns= not significant. (E) Four days after injections, effect of *TcKnk* dsRNA on transcript levels was determined by collecting dsRNA-treated insects (n=4)

from each treatment at the indicated stage of development. Depletion of transcripts was detected by extracting total RNA from each treatment followed by cDNA preparation and RT-PCR. *RpS6* transcript was used as an internal loading control. Numbers in parentheses indicate the cycle number for each of transcripts shown.

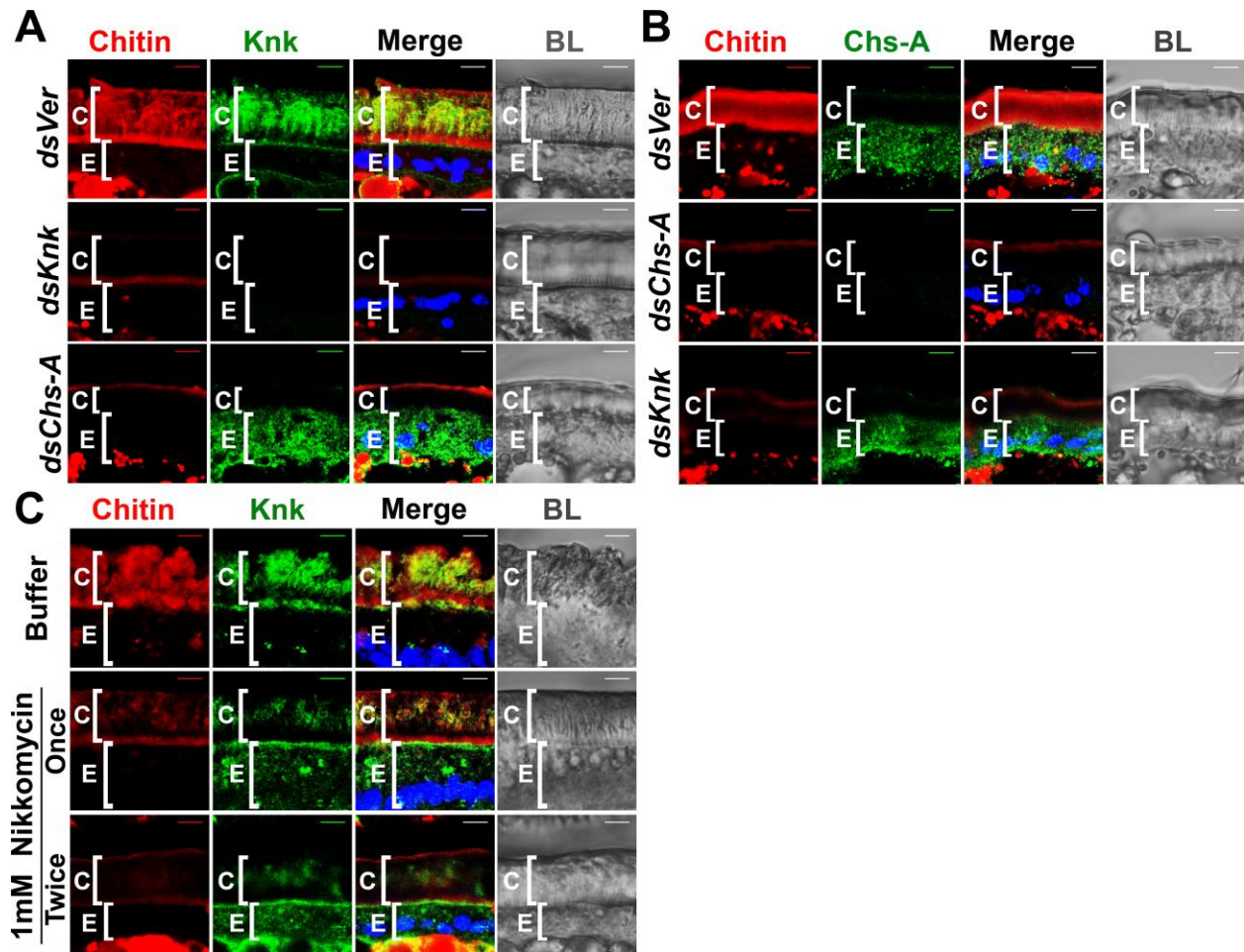


**Figure 2.5 TcKnk is a GPI-anchored, membrane-bound protein.**

Recombinant TcKnk protein expressed in Hi-5 cells infected with a recombinant baculovirus containing the ORF of TcKnk. After 72 h of infection, the medium was removed and fresh medium was added along with 100  $\mu$ l of phosphatidylinositol-specific phospholipase-C (PI-PLC) from *Bacillus cereus* (7.89 units/mg) for 4 h and the proteins in the medium and cell pellet were subjected to western blot analysis using an anti-Knk antiserum. Lanes: 1, Size marker; 2, Medium from TcKnk-expressing Hi-5 cells after 72 h of infection; 3, Cell pellet from TcKnk-expressing Hi-5 cells 72 h after infection. For lanes 4-7, old medium was removed and replaced with fresh medium with or without added PI-PLC. 4, Medium from TcKnk-expressing Hi5 cells after mock-treatment for 4 h without PI-PLC; 5, Medium from TcKnk-expressing Hi-5 cells 4 h after PI-PLC treatment; 6, TcKnk-expressing Hi-5 cell pellet without PI-PLC treatment; 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 lane 4 as well as lane 7). The two lower immunoreactive bands probably represent TcKnk protein without GPA anchor and/or unprocessed forms.

**Procuticular chitin associates with TcKnk and dictates its cellular distribution.**

Immunostaining of the lateral body wall of pharate adults with polyclonal anti-Knk antibodies revealed a layer of TcKnk at the junction between the inner layer of the endocuticle and the apical surface of the epidermal cells, as expected for a membrane-anchored protein. However, immunostaining was most intense throughout the newly synthesized chitinous procuticle, suggesting that TcKnk becomes incorporated into the cuticle after detachment from the plasma membrane by enzymatic cleavage of its GPI anchor (Fig 2.6A, top row).

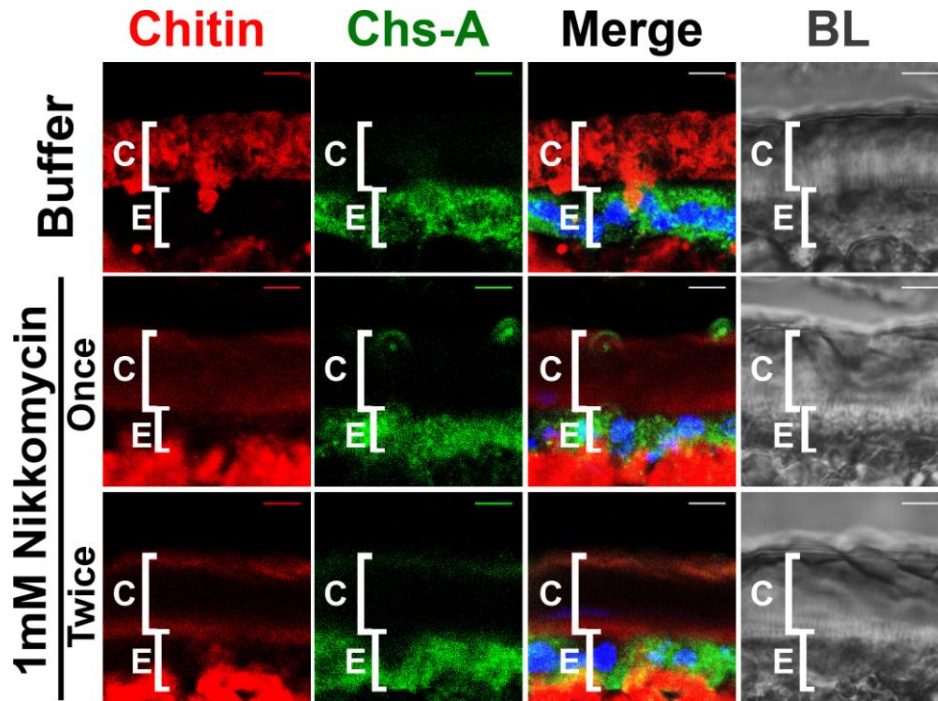


**Figure 2.6 TcKnk is mislocalized following disruption of chitin synthesis.**

(A) Cryosections of *T. castaneum* pharate adult lateral body walls (20  $\mu\text{m}$  thick) of control *TcVer* (*dsVer*), *TcKnk* (*dsKnk*) and *TcChs-A* (*dsChs-A*) dsRNA-treated insects were immunostained with *D. melanogaster* Knk (DmKnk) antiserum (green). (B) Distribution of TcChs-A was determined using anti-TcChs-A antibody (green) in various dsRNA-treated insects. Specificities of the DmKnk and TcChs-A antibodies as well as the “chitin-probe” were ascertained using insects subjected to RNAi for these two genes as controls. The punctate distribution of both TcChs-A and TcKnk within cells suggests vesicular localization. (C) Nikkomycin (1 mM, 0.2  $\mu\text{l}$ , per insect) was injected either once at the pharate pupal stage or twice (on d 1 and d 3 of the pupal stage) prior to cryosectioning and immunostaining for TcKnk (green) and staining for chitin (red). Chitin (red); Proteins (green); DAPI (blue); C, cuticle; E, epithelial cell. Scale bar = 5  $\mu\text{m}$ .



RNAi for TcChs-A prevented synthesis of cuticular chitin as expected, but unexpectedly also resulted in the mislocalization of the TcKnk protein, preventing its secretion and incorporation into the newly forming cuticle. Instead, the vast majority of TcKnk was retained within the epidermal cells (Fig 2.6A, bottom row). Conversely, down-regulation of *TcKnk* transcripts did not alter the cellular distribution of TcChs-A (Fig 2.6B, column 2).



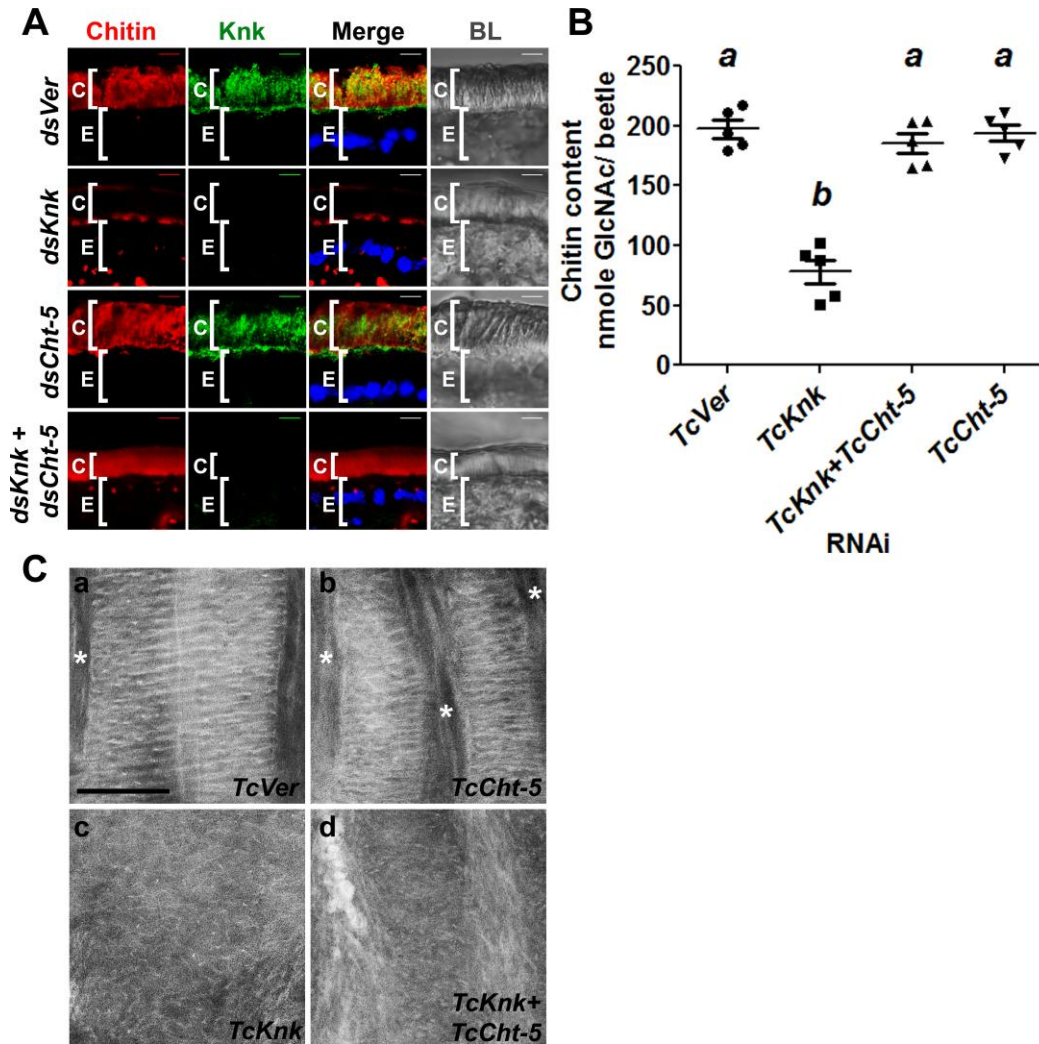
**Figure 2.7 Localization of TcChs-A in elytra of pharate adults after nikkomycin treatment.** Cellular distribution of TcChs-A (green) in insects injected once or twice with 0.5  $\mu$ l of 1 mM nikkomycin (a chitin synthase inhibitor; see Methods) was not changed in comparison with buffer-injected insects after analysis by confocal microscopy (as described in Fig 2.6). Red, Rhodamine-conjugated chitin binding probe; C, cuticle; E, Epithelial cells. Scale bar = 5  $\mu$ m.

Based on these observations we hypothesized that TcChs-A may facilitate the proper targeting of TcKnk to the new cuticular chitin, either through direct protein-protein interaction(s) or via its product, chitin. To distinguish between these two possibilities, we inhibited chitin synthesis at the pharate adult stage by administering lethal doses of nikkomycin, a structural analog of the UDP-GlcNAc substrate of chitin synthases, and monitoring its effect on TcKnk trafficking. We

reasoned that nikkomycin would specifically inhibit the production of chitin by TcChs-A without perturbing any possible protein-protein interactions between TcChs-A and TcKnk. As expected, nikkomycin-treated insects had progressively diminished levels of chitin in the procuticle, whereas the level and cellular distribution of TcChs-A was unaffected (Fig 2.7). Importantly, TcKnk was mislocalized (retained) within the epidermal cells (Fig 2.6C, column 2), just as we had observed following TcChs-A RNAi. These results suggest that TcChs-A has no direct involvement in the normal secretion of TcKnk or its incorporation into the extracellular cuticular matrix, but rather, that the presence of extracellular chitin itself is a precondition for the cuticular localization of TcKnk in the procuticle.

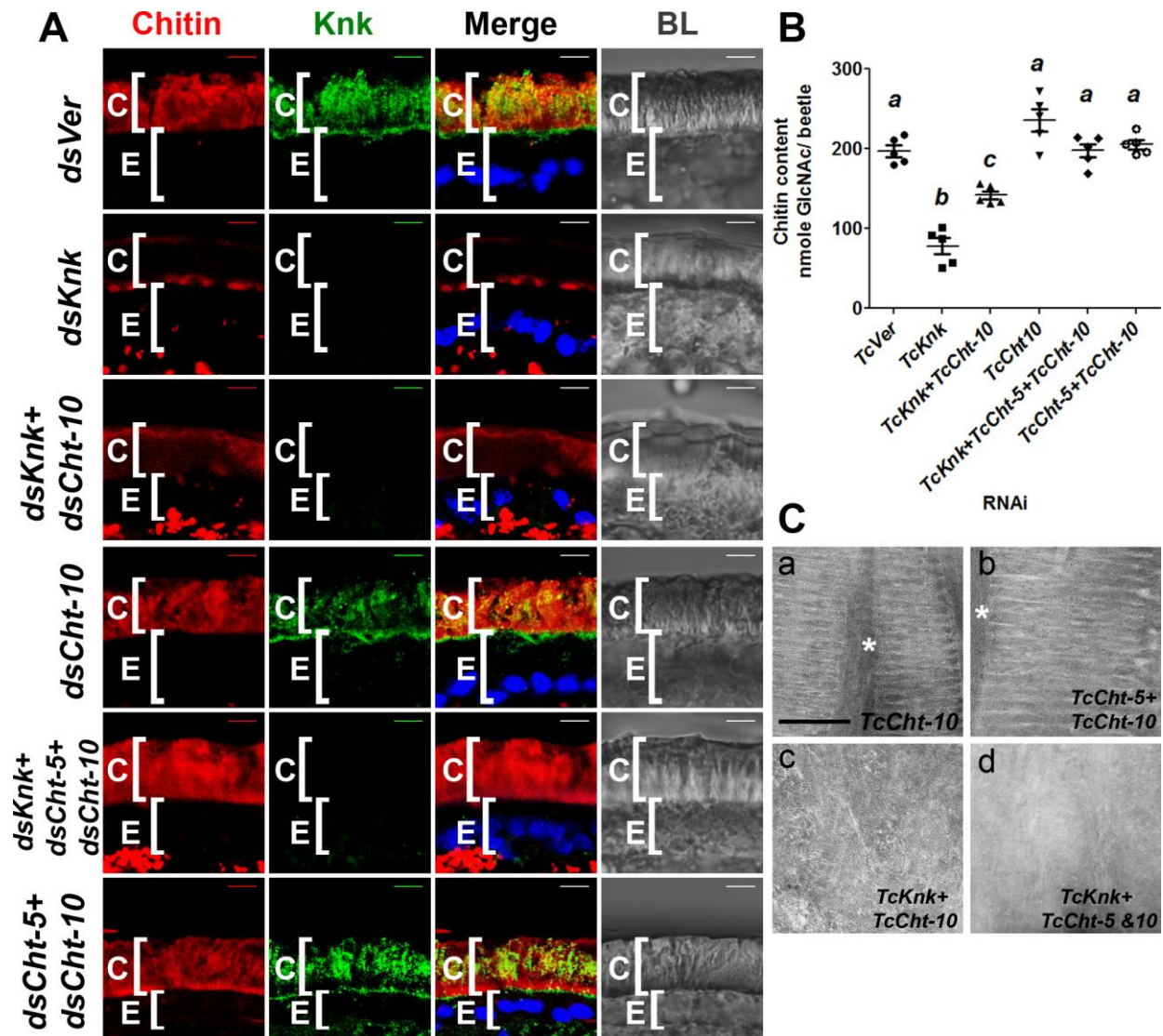
### **TcKnk protects cuticular chitin from degradation by chitinases and organizes chitin into laminae.**

Since the reduction in chitin content brought about by RNAi for TcKnk could not be attributed to altered levels or distribution of TcChs-A (Fig 2.6B), we suspected that TcKnk might have a previously unrecognized function in protection of nascent cuticle from molting fluid, a role previously attributed exclusively to the envelope. We performed TcKnk RNAi and assessed whether the resulting chitin depletion could be rescued by simultaneous down-regulation of transcripts for either or both of the two chitinase-encoding genes *TcCht-5* and *TcCht-10* as these two genes have been shown to be critical for molting and turnover of chitin in the old cuticle (Zhu et al., 2008b). Remarkably, in agreement with the above hypothesis confocal microscopy and quantitative chitin content analysis revealed that chitin levels could be restored to normal *TcVer* control when both *TcCht-5* and *TcCht-10* were down regulated along with *TcKnk* (Fig 2.8A-B and Fig 2.9A-B). RNAi of *TcCht-5* alone was more effective than down-regulation of *TcCht-10* in rescuing dsRNA-*TcKnk*-mediated chitin depletion, indicating a more critical role for *TcCht-5* in chitin turnover (Fig 2.8A, row 4; Fig 2.8B).



**Figure 2.8 TcKnk aids laminal organization of the procuticular chitin and prevents its degradation by molting fluid chitinases.**

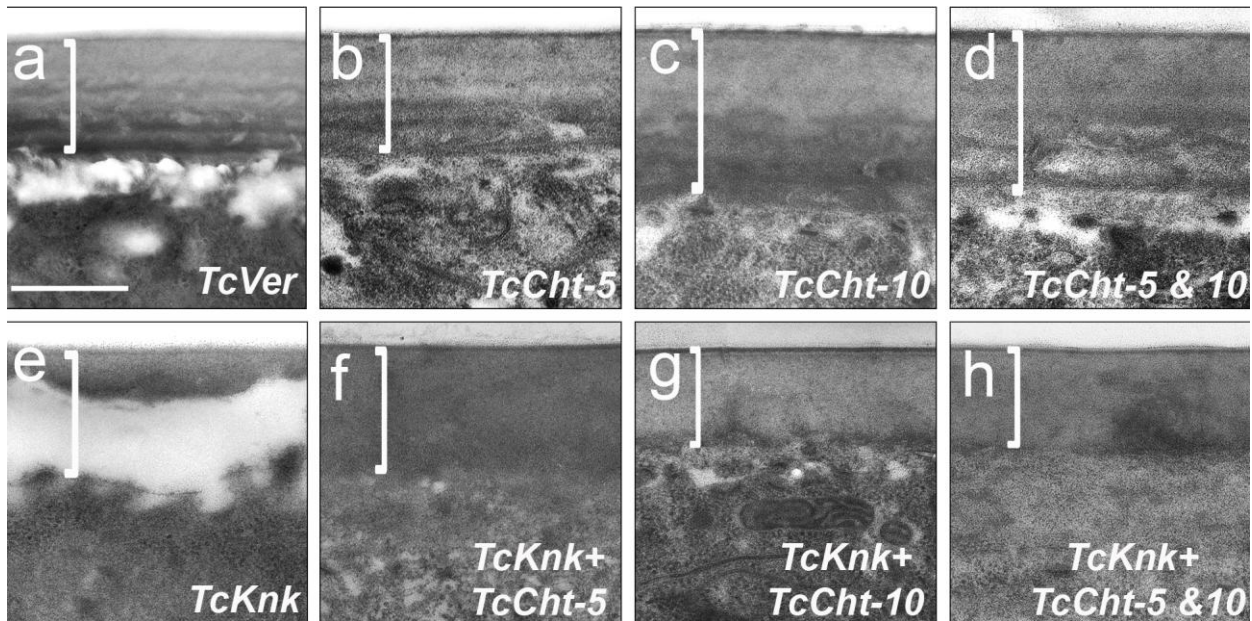
(A) Chitin depletion resulting from *TcKnk* RNAi was reversed following double RNAi for *TcKnk* and *TcCht5* (*dsKnk+dsCht-5*). Chitin (red); TcKnk (green); DAPI (blue); C, cuticle; E, epithelial cell. Scale bar = 5  $\mu$ m. (B) Analysis of total chitin content by a modified Morgan-Elson assay and reported as mean  $\pm$  SE (n = 5 each). Statistical significance was computed by the Student's *t*-test. Means identified by different letters are significantly different at  $p < 0.05$ . (C) Ultrastructure of pharate adult elytral cuticle. Elytra from *Ver* dsRNA-treated insects (*TcVer*) consist of horizontal brick-wall like chitin laminae with respect to the apical surface of epithelial cells that are occasionally separated by vertical channels (\*, perpendicular to the apical plasma membrane) that contain a fibrous material, probably chitin (a). Cuticle after *TcCht-5* (b) dsRNA-treatment is normal (*TcVer* RNAi). The elytral cuticle of pharate adults treated with dsRNA for *TcKnk*, by contrast, is disorganized (c). Concomitant depletion of *TcCht-5* (d) along with *TcKnk* transcripts does not restore the laminal organization of chitin. Scale bar for a, b, c and d = 500 nm.



**Figure 2.9 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 cell. Scale bar = 5  $\mu$ m. (B) Analysis of total chitin content by a modified Morgan-Elson assay and reported as mean  $\pm$  SE (n = 5 each). Statistical significance was computed by the Student's *t*-test. Means identified by different letters are significantly different at  $p < 0.05$ . (C) Ultrastructure of pharate adult elytral cuticle. Cuticle after *TcCht-10* (a) single or *TcCht-5* and *TcCht-10* double (b) dsRNA-treatment is indistinguishable from *TcVer* dsRNA treated samples (see Fig 2.8C). The elytral cuticle of pharate adults treated with dsRNA for *TcCht-5* and *TcCht-10* along with dsRNA for *TcKnk* (c and d) does not restore the laminar organization of chitin. Scale bar for a, b, c and d = 500 nm.

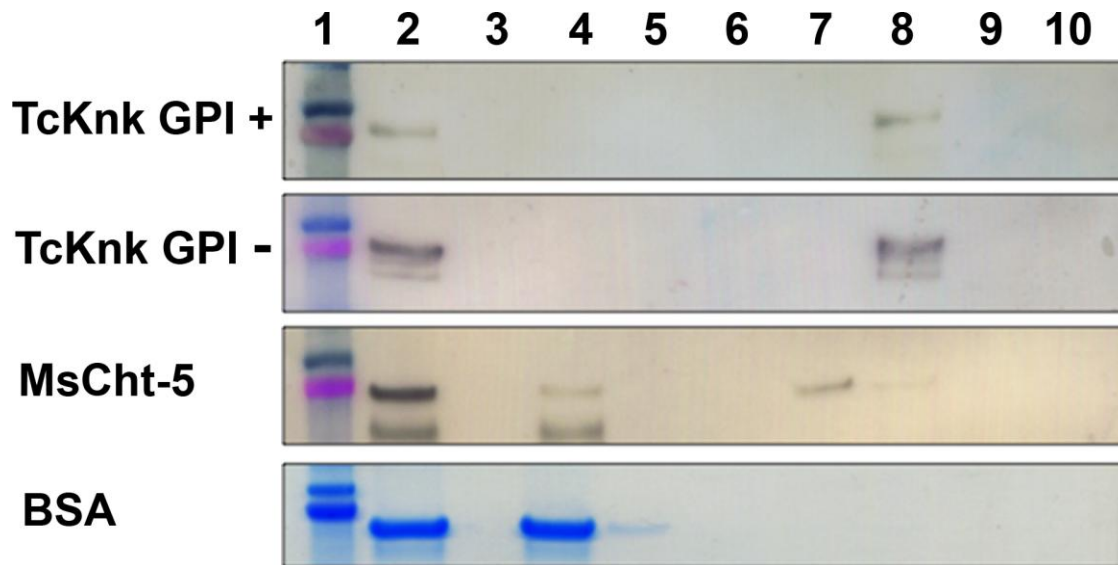
Transmission electron microscopy of the adult elytral cuticle additionally indicated a role for TcKnk in the organization of cuticular chitin as down-regulation of *TcCht-5* and *TcCht-10* along with *TcKnk* failed to rescue the normal laminar architecture of the elytral procuticle even though chitin content was restored to normal levels (*TcVer* control RNAi) (Fig 2.8C and Fig 2.9C). These results suggest that TcKnk is essential for the laminar organization of chitin in the elytral procuticle. Similar results were also observed for the ventral body wall cuticle, which has fewer nascent laminae (Fig 2.10).



**Figure 2.10 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* knock-down, in the ventral body wall cuticle, the laminae are absent (e). Simultaneous knock-down of *TcCht-5* (f), *TcCht-10* (g) or *TcCht-5* and *TcCht-10* (h) along with *TcKnk* transcripts does not restore laminar structure even though chitin levels are restored (see Fig 2.8 and Fig 2.9). Scale bar for a, b, c, d, e, f, g and h= 500 nm.

It is conceivable that TcKnk directly interacts with chitin to enable its laminar organization. To test this hypothesis, recombinant TcKnk (with or without the GPI anchor) was extracted from the membrane fraction of Hi-5 cells expressing TcKnk either directly or by PI-PLC treatment, and

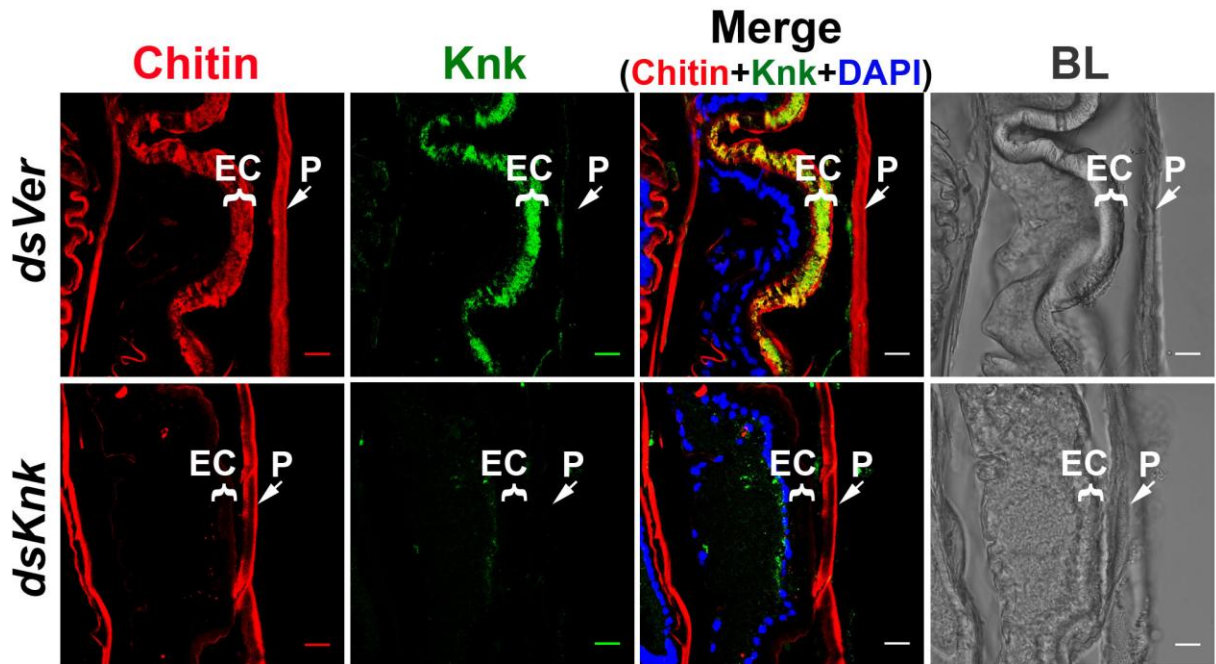


**Figure 2.11 Colloidal chitin-binding assay of recombinant TcKnk, chitinase and bovine serum albumin.**

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 using the Mem-Per kit (TcKnk-GPI+) or released by treatment with PI-PLC (TcKnk GPI-). *M. sexta* chitinase-5 (MsCht-5) recombinant protein was purified as described previously (13). Proteins used for the chitin-binding assay are indicated on the left. Lanes: 1, size markers; 2, protein sample as indicated on the left; 3, colloidal chitin used for the binding assay; 4, flow-through fraction; 5, 10 mM sodium phosphate buffer, pH 8.0 wash; 6, 10 mM sodium phosphate buffer containing 1 M NaCl, pH 8.0 wash; 7, 1% Calcofluor eluate; 8, protein eluted by boiling the colloidal chitin with bound proteins for 10 min in 50 µl of 1 X SDS-PAGE sample buffer; 9, negative control (uninfected Hi-5 cells after PI-PLC treatment or proteins extracted from them by Mem-Per kit); 10, proteinase inhibitor cocktail which 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 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 as 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 panel). BSA was used as a negative control for non-specific binding of proteins to chitin.

was assayed for binding to colloidal chitin as previously described for *M. sexta* chitinase (Arakane et al., 2003). Both forms of TcKnk bound to colloidal chitin more tightly than did *M. sexta* chitinase-5, which has a peritrophin A-type chitin-binding domain. Chitin-bound TcKnk could not be eluted with Calcofluor, but required treatment with hot SDS for dissociation from colloidal chitin (Fig 2.11). The high affinity of TcKnk for chitin, its co-localization with chitin in the cuticle and its ability to prevent cuticle degradation by chitinases strongly suggest a critical role for this protein in cuticular chitin maintenance during the molting process.

Since the old endocuticular chitin must be partially digested by chitinases to promote shedding of the old cuticle as the exuvium during a molt, one might expect a depletion of TcKnk in the old cuticle at the time of apolysis. Indeed, sections of pharate adults that contain both old pupal cuticle and new elytral cuticle clearly reveal localization of TcKnk predominantly in the new cuticle (Fig 2.12). The substantial reduction of TcKnk in the old cuticle indicated by the immunostaining may account for the susceptibility of the old cuticle to molting fluid chitinolytic enzymes. It is unknown what factor or factors account for the disappearance of detectable TcKnk in mature cuticle. Its depletion might be due to digestion by molting fluid proteases. In that regard, it is interesting to point out that RNAi of transcripts for a chymotrypsin-like protein that is present in cast cuticles results in molting defects (Broehan et al., 2010). Immunolocalization of the proteolytic enzyme(s) involved in cuticular protein turnover will lend further support to our conclusion that the envelope alone cannot account for the stability of the newly synthesized cuticle in the presence of molting enzymes. An alternative explanation, that cuticle sclerotization reduces the permeability of the cuticle to Knk antibodies, seems unlikely because chitinase-5 can be detected in old pupal cuticle by immunostaining (see Fig 2.1).



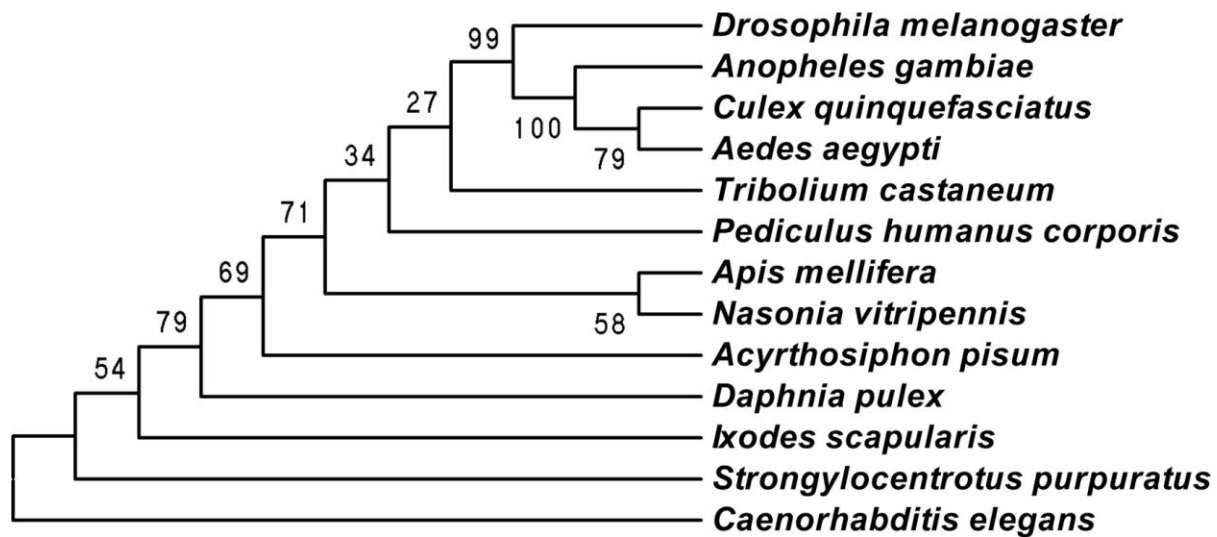
**Figure 2.12 TcKnk is preferentially detected in the new cuticle.**

Chitin staining (red) of cryosections of pharate adult insects containing both the old pupal cuticle (P) and the newly-synthesized elytral cuticle (EC). TcKnk (green) is only detected in the new elytral cuticle. Nuclei are stained with DAPI (blue). Scale bar = 10  $\mu$ m. Insects subjected to treatment with dsRNA for *TcKnk* were included to demonstrate the specificity of the Knk antibody.

**Knk is widely distributed in invertebrates with chitinous extracellular matrices.**

TcKnk orthologs are present in all animal groups examined that secrete chitinous extracellular matrices, including representative species of insects, ticks, crustaceans, echinoderms and nematodes (Fig 2.13). Fungi are the only eukaryotes with chitinous extracellular matrices that lack TcKnk orthologs. All of these putative Knk proteins have the same domain organization, which is suggestive of an essential conserved function in those taxa.





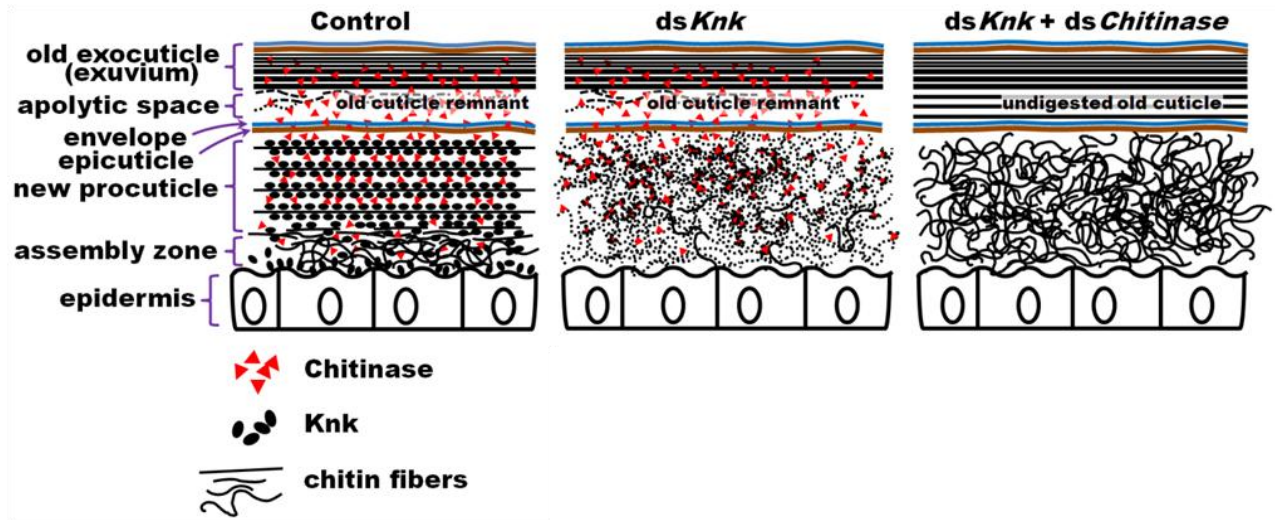
**Figure 2.13 Phylogenetic analysis of Knickkopf-like proteins from different taxa.**

Phylogenetic analysis of Knk homologues from arthropods including several orders of insect species, sea urchin, as well as *C. elegans* as an outlier was done using MEGA 4.0 (Tamura et al., 2007). Bootstrap analysis of 5,000 replications was carried out on trees inferred from the neighbor joining method. Bootstrap values are shown in the cladogram at the branching points. *Drosophila melanogaster* (fruit fly) (NP\_649981.1); *Anopheles gambiae* (African malaria mosquito) (XP\_313797.4); *Aedes aegypti* (Yellow fever mosquito) (XP\_001657332.1); *Culex quinquefasciatus* (Southern house mosquito) (XP\_001863519.1); *Pediculus humanus corporis* (head louse) (XP\_002430062.1); *Tribolium castaneum* (red flour beetle) (AEM60136.1); *Apis mellifera* (Honey bee) (XP\_394084.4); *Nasonia vitripennis* (parasitic wasp) (XP\_001606352.1); *Acyrtosiphon pisum* (pea aphid) (XP\_001950825.2); *Daphnia pulex* (water flea) (EFX88131.1); *Ixodes scapularis* (deer tick) (XP\_002407022.1); *Strongylocentrotus purpuratus* (sea urchin) (XP\_001187728.1); *Caenorhabditis elegans* (roundworm) (NP\_508959.1).

## Concluding remarks

Our work has revealed essential and unexpected roles for TcKnk during the exoskeletal molting process. This protein co-localizes with nascent chitin and organizes it into laminae to confer selective protection of the new cuticle from degradation by chitinases, while simultaneously allowing digestion of chitin in the inner layers of the old procuticle that enshrouds it. This discovery casts strong doubt on the long-held hypothesis that an impermeable envelope is sufficient to ensure this selective susceptibility of old versus new cuticle to molting enzymes. A model representing the dynamic changes in chitin degradation and deposition in the old and

newly forming insect cuticle, respectively, during molting including the role of Knk and chitinases is presented in Fig 2.14. The mechanism described herein is probably conserved in all chitinous invertebrates.



**Figure 2.14 Model of chitin deposition and degradation in the composite insect cuticle during molting.**

Note that chitinases are present in both the old exocuticle and the newly forming cuticle. The envelope and epicuticle layers do not exclude chitinases from the chitinous procuticle. Knk protects chitin from chitinases by acting as a chitin-binding protein and masking the chitin. Knk also facilitates formation of laminae by an unknown mechanism. Note the absence of Knk protein in the old exocuticle, presumably as a result of turnover by molting-associated proteases.

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**Chapter 3 - Retroactive promotes trafficking of  
the chitin-binding protein Knickkopf into the  
procuticle of *Tribolium castaneum***

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## Abstract

Deletion mutants of two genes, knickkopf (knk) and retroactive (rtv), were shown to have cuticular defects and embryonic lethality in *D. melanogaster*. Both, knk and rtv mutant embryos showed “blimp” phenotype similar to krotzkopf verkehrt (kkv) mutants (an ortholog of *Tribolium* chitin synthase A) indicating their role in cuticle integrity. Our recent study in *T. castaneum* has revealed an essential role for Knk protein in organization and protection of newly synthesized chitin from degradation by chitinases. An ortholog of *Drosophila* Rtv is detected *T. castaneum*, which we have named TcRtv. Confocal analysis showed localization of TcRtv predominantly inside the epidermal cells. RNA interference (RNAi) study for *TcRtv* showed similar molting defects and chitin depletion phenotype like *TcKnk* and *TcChs-A* dsRNA treatment. Co-injection of dsRNAs for *TcRtv* along with *TcCht-5* rescued the chitin depletion phenotype, indicating a role for TcRtv in protecting chitin from chitinases. In absence of TcRtv, TcKnk protein was mislocalized from the procuticle to within the epithelial cells indicating important role for TcRtv in proper trafficking of TcKnk to the procuticle where the latter protein associates with and protects chitin. Our data demonstrates that TcRtv indirectly promotes cuticle synthesis and organization by facilitating TcKnk’s localization in the procuticle.

## **Introduction**

A critical feature of the insect molting process is the simultaneous synthesis and degradation of chitin at different sites within the procuticle (Locke, 2001; Reynolds and Samuels, 1996a). Chitinolytic enzymes (molting fluid chitinases and N-acetylglucosaminidases) dissolve parts of the old chitinous exoskeleton into oligomeric and monomeric N-acetylglucosamine (GlcNAc) units, respectively, and subsequently recycle them into the collective pool of biosynthetically derived activated precursors of GlcNAc monomers (UDP-GlcNAc) used for the synthesis of new cuticular chitin (Merzendorfer, 2006; Noble-Nesbitt, 1963) (Reynolds and Samuels, 1996). The enzyme that catalyzes the addition of these monomers onto the growing chitin oligosaccharide is the integral membrane-bound chitin synthase (Chs) (Arakane et al., 2005; Merzendorfer, 2006; Merzendorfer and Zimoch, 2003). There are two functionally specialized Chs isoforms, Chs-A and Chs-B, that are responsible for the synthesis of chitin in epidermal and midgut epithelial cells, respectively. The products of these two enzymes are organized into completely different extracellular matrices, namely the cuticle and the peritrophic matrix (PM). Cuticular chitin directly overlies the epidermal cell surface and is a highly ordered structure composed of helicoidal stacks of laminae, the latter consisting of parallel arrays of chitin fiber bundles. In contrast, the PM has a less ordered, lattice-like structure, is hydrophilic and porous, and is physically separated from the midgut epithelium that secretes it. These two forms of chitinous extracellular matrices also differ in the nature and properties of their constituent proteins, further contributing to the unique attributes of each.

Upon synthesis and secretion into the extracellular (subcuticular) space by epidermal cells, nascent chitin spontaneously aggregates into microfibrils and accumulates within the assembly zone (the layer immediately above the apical surface of the epidermal cells where nascent layers of the procuticle form) to eventually organize into the new cuticular laminae (Moussian, 2010; Moussian et al., 2006a). At the ultrastructural level, the new cuticle extends from its inner boundary on the apical side of the epidermal cell membrane to its outer surface “envelope” layer that appears to partition it from the overlying old procuticle (Reynolds and Samuels, 1996b). During the molting process the outer parts of the old cuticle (including the envelope, epicuticle and exocuticle) are shed as the exuvium, while the less sclerotized endocuticle is digested by chitinolytic and proteolytic enzymes of the molting fluid that accumulate in the apolytic space. Near the time of appearance of molting fluid droplets in the



apolytic space, a fresh layer of waxy envelope is secreted by epidermal cells. This newly deposited envelope layer becomes the outermost (superficial) layer of the new cuticle. The long-held notion that the hydrophobic envelope protects chitin in the newly forming procuticle was disproved by our recent study on cuticulogenesis in the red flour beetle, *Tribolium castaneum*. We showed that the envelope layer functions only as a structural boundary at the early stages of molting, and does not function as a barrier against molting fluid chitinases (Chaudhari et al., 2011). Indeed, the molting fluid chitinase of *T. castaneum* (TcCht-5) was shown to be present throughout the new procuticle and to co-localize with chitin. The mysterious stability of the new cuticle in intimate contact with enzymatically active chitinases as old cuticle is being degraded was shown to be due to the physical association of the cuticle assembly protein, Knickkopf (TcKnk; homologue of *Drosophila melanogaster* knickkopf, DmKnk). This protein also was found to be required for the laminar organization and stabilization of chitin in the new procuticle (Chaudhari et al., 2011).

Like DmKnk, the protein retroactive (DmRtv) was also previously demonstrated to affect cuticle differentiation and tracheal tube-length morphogenesis involving the following biological processes: regulation of tube diameter, tube architecture and chitin-based embryonic cuticle biosynthesis (Moussian et al., 2005; Moussian et al., 2006b; Ostrowski et al., 2002). DmRtv is predicted to form a membrane-anchored, three-finger loop structure that interacts with chitin via two aromatic residues situated on each loop (Moussian et al., 2005). Although a direct role for this protein in cuticular chitin filament organization in embryonic tracheal tubules of *D. melanogaster* was envisaged, the mechanism of this ordering process is unclear. In the current study we show that a direct effect of the *T. castaneum* Rtv homologue (TcRtv) on cuticular chitin organization is unlikely, given its predominant intracellular distribution within epidermal cells. Instead, its activity is shown to be crucial for the proper trafficking of TcKnk to the procuticle where the latter protein associates with and protects chitin. Hence, TcRtv indirectly promotes cuticle synthesis and organization by facilitating TcKnk's localization in the integument.

## **Materials and methods**

### **Insect cultures**

The GA-1 strain of *T. castaneum* was used for all experiments. Insects were reared at 30°C in wheat flour containing 5% brewer's yeast under standard conditions as described previously (Beeman and Stuart, 1990; see Chapter 2).

### **Identification of the *TcRtv* gene in the *T. castaneum* genome database**

An extensive, genome-wide search for homologs of *DmRtv* in the *T. castaneum* genome database was performed using NCBI programs TBLASTN and BLASTP using the amino acid sequence of DmRTV as query. This search resulted in the identification of an orthologous gene, which we have named *T. castaneum retroactive* (*TcRtv*).

### **Cloning and sequencing of a *TcRtv* cDNA**

A DNA fragment containing the complete coding sequence of *TcRtv* (453 bp) was amplified by reverse transcriptase-PCR (RT-PCR) using the gene-specific primers (forward primer 5'-ATGGGTCTGTTTAGATCAATTT-3' and the reverse primer 5'-AGACTGATTTTACGATTTTTGTAA-3') using cDNA prepared from the RNA extracted from different stages of beetle development as template. The amplified fragment was cloned into the pGEMT vector. Sequencing of the cDNA clone was carried out at the DNA sequencing facility at Kansas State University. The accession number for the *TcRtv* protein is EFA01783.1.

### **Phylogenetic analysis of *TcRtv***

Rtv-like proteins were identified by TBLASTN searches of the fully sequenced genomes of *T. castaneum*, *Anopheles gambiae*, *Aedes aegypti*, *Culex quinquefasciatus*, *Bombyx mori*, *Acyrtosiphon pisum*, *Nasonia vitripennis* and *Ixodes scapularis* using the amino acid sequence of DmRtv as query. Multiple sequence alignments of *TcRtv* and the related Rtv-like proteins from insects and arthropods were carried out using the ClustalW software prior to phylogenetic analysis. A consensus phylogenetic tree was constructed using MEGA 4.0 neighbor joining method with 5000 replications (Tamura et al., 2007).

## Determination of expression profiles of *TcRtv*

The RNeasy Mini kit (Qiagen) was used according to the manufacturer's instructions to isolate RNA from embryos, larvae, pharate pupae, pupae, young adults and mature adults (n=4) (Arakane et al., 2005; see Chapter 2). For determination of tissue specificity of expression, RNA was extracted from pools of anterior midgut, middle midgut, posterior midgut and carcass (the whole body without gut) of larvae (n = 10) at the penultimate or last instar larval stages. The Superscript III first-strand cDNA synthesis system (Invitrogen) was used according to the manufacturer's instructions to synthesize first-strand cDNA using RNA extracted from different tissues or developmental stages as template. These cDNA templates and the pair of *TcRtv*-specific primers, forward primer 5'-ATGGGTCTGTTTAGATCAATTT-3' and reverse primer 5'-AGACTGATTTTACGATTTTGTAA-3', were used for RT-PCR to determine the *TcRtv* expression profile. The *T. castaneum ribosomal protein-6 (TcRpS6)* gene was used as an internal control for equal loading of cDNA templates (Arakane et al., 2011).

## RNA interference studies

Two different regions of the *TcRtv* gene were selected for making dsRNAs. Pairs of forward and reverse primers for the chosen regions (Table 3-1) were used to amplify the dsRNA sequences by using the cloned *TcRtv* cDNA as template. The Ampliscribe T7-Flash Transcription Kit

**Table 3.1 Primer sequences for generation of dsRNAs.**

dsRNA	Sequence (5'-3')	Nucleotide positions	dsRNA Length, bp
1	<u>F= TAATACGACTCACTATAGGGTGGAAATTCTCAAACGC</u> R= TAATACGACTCACTATAGGGTAGAGACAGAAAATCC	72-233	161
2	<u>F= TAATACGACTCACTATAGGGTTTGTATGCCGATCTC</u> R= TAATACGACTCACTATAGGGTCTGCCGAGGAGACTT	91-365	274

(Epicentre Technologies) was used to synthesize dsRNA as described previously (Arakane et al., 2005). dsRNA for the tryptophan oxygenase, *dsVermilion* (*dsVer*) gene, which is required for eye pigmentation, was used as a control. *dsRNA TcRtv* or *dsRNA TcVer* were injected into animals in the young larval, last instar larval or pharate pupal stages of *T. castaneum* development (200 ng per insect, n=30). Five insects were collected at the young larval, pharate pupal and pharate adult stages of development 3-5 d after dsRNA treatment. Total RNA was extracted from the collected insects for measuring transcript levels by RT-PCR using gene-specific primer-pairs.

### **Expression of recombinant TcRtv**

The full-length *TcRtv* cDNA clone was used as template to amplify the complete coding region of the *TcRtv* gene. A primer pair containing appropriate restriction enzyme sites [forward primer 5'-TATCCCGGGATGGGCTGTTTAGATC-3' (Sma I) and a reverse primer 5'-CAGACTGATTTTACGATTTTTGTAATCTAGATAT-3' (Xba I)] was used to facilitate directional cloning of the *TcRtv* open reading frame (ORF) DNA in the pVL1393 baculovirus expression vector (BD Pharmingen). PCR-amplified, full length *TcRtv* DNA and the pVL1393 vector DNA were digested with the same pair of restriction enzymes and ligated as described previously (Zhu et al., 2008a). Recombinant baculovirus for expression of TcRtv protein was constructed using BaculoGold DNA and 1 µg of the recombinant baculovirus transfer vector pVL1393 DNA containing the *TcRtv* ORF, and was amplified to obtain a high titer virus. Hi-5 cells (*Trichoplusia ni* cell line) were used to express the TcKnk protein as described earlier (Zhu et al., 2008a).

### **Immunohistochemistry**

*TcRtv* dsRNA-treated pharate adult (one day prior to adult eclosion) insects were collected (n=5) and fixed as described previously (Chaudhari et al., 2011). Cryosections of 20 µm thickness were made and stained for specific proteins using chicken antiserum to *T. castaneum* Rtv (1:100), rabbit antiserum (1:100) to *D. melanogaster* Knk and rabbit antiserum to *T. castaneum* Chs-A (1:50) as primary antibodies. Alexa 488 goat-anti-chicken IgG (1:1000, Ex/Em=495nm/519nm) and Alexa 488 goat-anti-rabbit IgG (1:1000, Ex/Em=495nm/519nm) were used as secondary antibody for green fluorescence detection of the respective proteins. Confocal microscopy was

performed on an LSM META 510 laser scanning confocal microscope as described in Chapter 2 materials and methods.

### **Chitin content analysis**

Following dsRNA-treatment, pharate pupae and pharate adults of *T. castaneum* were collected for chitin content analysis by a modified Morgan-Elson method as described previously (Arakane et al., 2005). *TcVer* dsRNA and *TcChs-A* dsRNA-treated insects were used as negative and positive controls for the experiment, respectively.

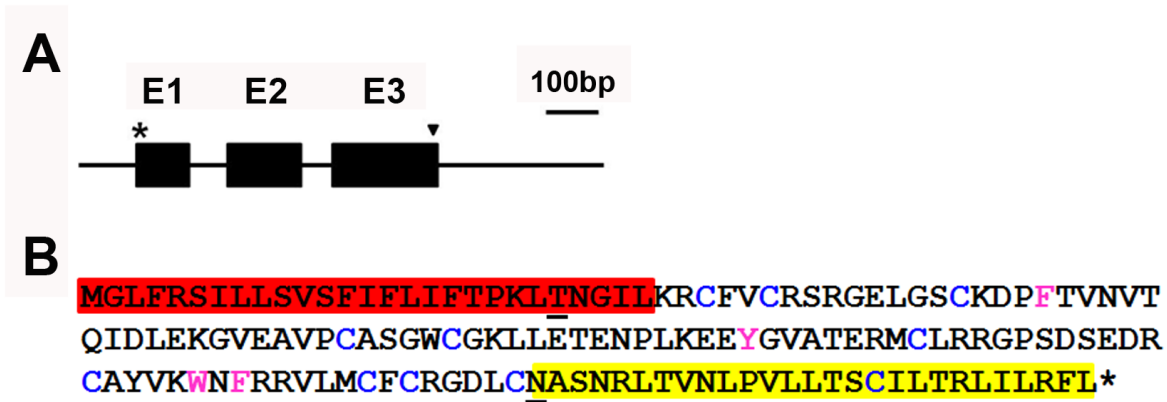
### **Transmission electron microscopic (TEM) analysis**

Pharate pupae were injected with dsRNA and pharate adult samples were collected on pupal day 5 and fixed and embedded in EMBED 812/ Araldite resin as described previously (Chaudhari et al., 2011). Resin embedded samples were then thin-sectioned (silver to gold section) and imaged on a CM-100 TEM (FEI Co.).

## **Results**

### **TcRtv is a conserved protein required for insect molting**

TBLASTN and BLASTP searches of the *T. castaneum* genome using the DmRtv protein sequence as query identified only a single gene coding for an orthologous protein. TcRtv maps on LG4, position 24.3 cM. This *T. castaneum* Retroactive gene (*TcRtv*) is composed of three exons and encodes a 150-residue long protein with a predicted C-terminal GPI anchor and a  $\omega$ -asparagine at position 124 (Fig 3.1 A, B). A predicted cleavable signal peptide at its N-terminus suggests that this protein enters the ER secretory pathway prior to GPI-anchoring and is transported to the cell surface via intracellular secretory vesicles. Developmental expression analysis revealed the presence of *TcRtv* transcripts at all stages of beetle development including embryonic, larval, pupal and adult (Fig 3.2A). Lower levels of TcRtv expression were detected at the embryonic and young larval stages relative to later stages of development. Tissue-specific expression of TcRtv is likely to be of epidermal origin, as its expression could only be detected in the carcass and not in the anterior, middle or posterior midgut tissues (Fig 3.2B).

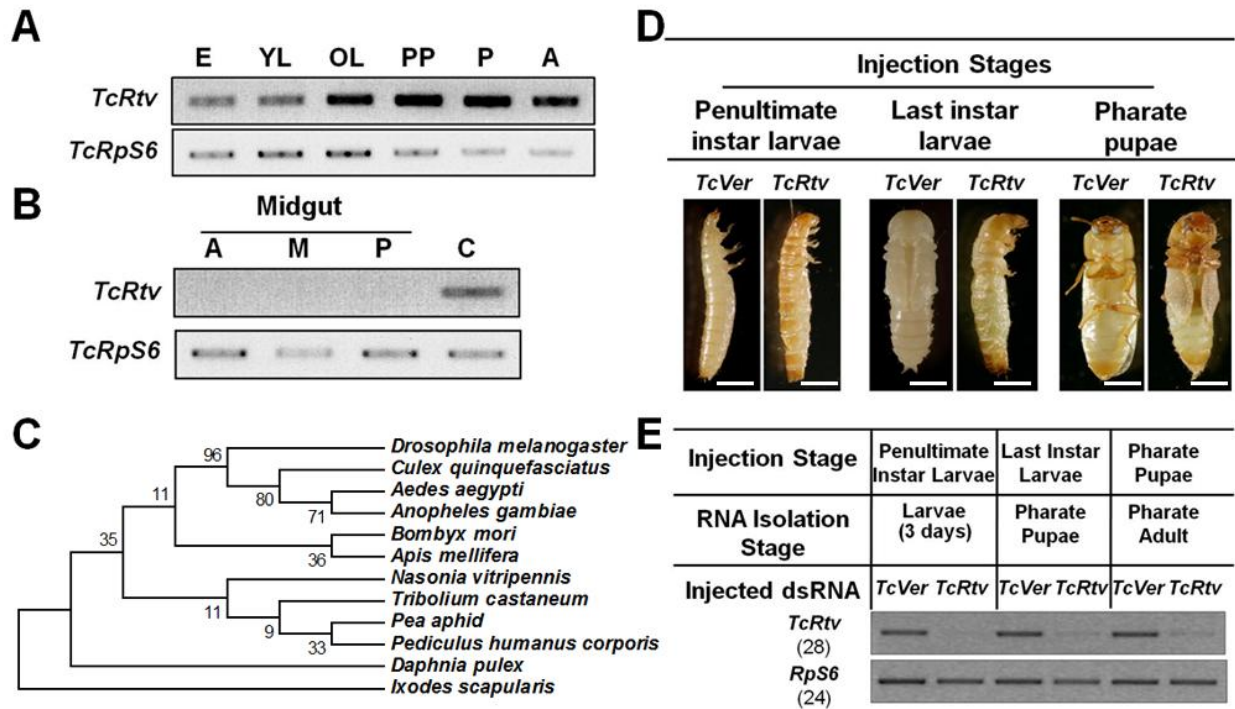


**Figure 3.1 Exon-intron and domain organization of TcRtv.**

(A) Schematic diagram of the exon–intron organization of TcRtv gene. The exon–intron organization of TcRtv 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 three exons. Closed triangle and \* indicates start and stop codons, respectively. (B) TcRtv encodes a 15 kDa protein with an N-terminal signal peptide (red) and a C-terminal hydrophobic region (Yellow). Ten conserved cysteine and aromatic residues are shown in blue and pink color, respectively. The ω-asparagine residue where the cleavage is predicted to occur for GPI anchoring is underlined.

When other fully sequenced arthropod genomes were queried with DmRtv or TcRtv sequences, only a single homolog was identified in all the insect species examined as well as in other arthropods such as ticks. The presence of an ortholog of TcRtv gene in all sequenced arthropod genomes, suggests an essential function for this protein in arthropod survival and development (Fig 3.2C).

Given the ubiquitous presence of *TcRtv* transcripts during insect growth, we hypothesized a critical role for this protein in post-embryonic development. To test this hypothesis, we depleted *TcRtv* transcripts via RNA interference (RNAi) by administration of *TcRtv*-specific dsRNA (dsTcRtv). RNAi of *TcRtv* led to molting arrest at larval-larval, larval-pupal or pupal-adult stages of post-embryonic development, depending upon stage at injection, with significant reductions in transcript levels at each stage tested (Fig 3.2 D-E). Apolysis and slippage of the old cuticle proceeded at the subsequent molt after injection of *TcRtv* dsRNA into penultimate-instar larvae, but the larvae failed to complete the larval-larval molt and remained entrapped within their old larval cuticle. Similarly, insects arrested at the larval-pupal or pupal-



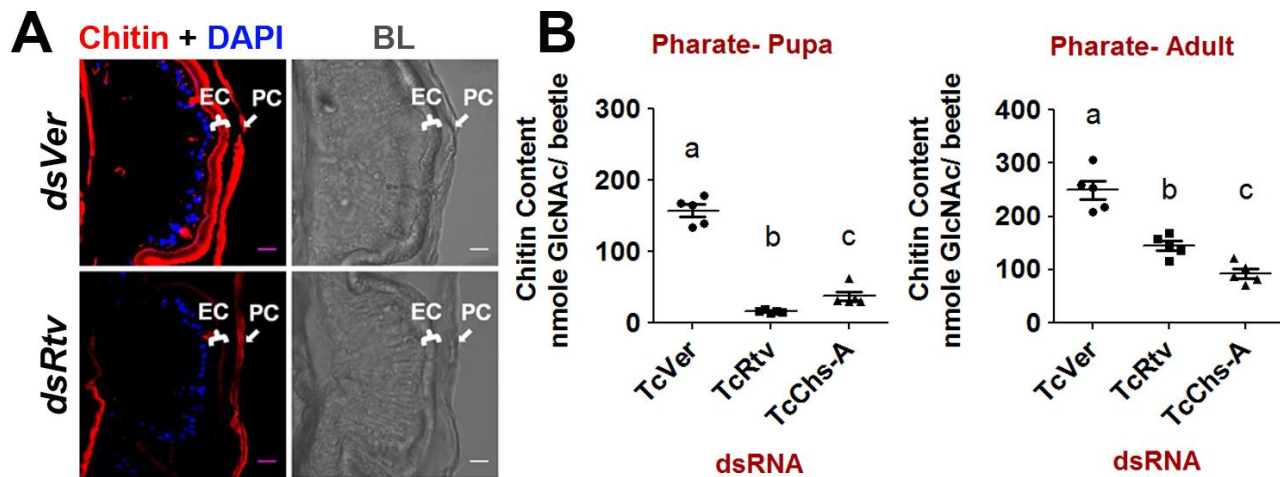
**Figure 3.2 TcRtv is a conserved protein required for insect molting.**

(A) Expression of *TcRtv* during *Tribolium* development. Total RNA was extracted from different stages of development: E, eggs; YL, young larvae; OL, old last instar larvae; PP, pharate pupae; P, pupae and A, adult. RT-PCR (28 cycles) was performed using cDNA prepared from total RNA and gene-specific primers. *TcRpS6* (*T. castaneum* ribosomal protein-S6) was used as an internal loading control (24 cycles). (B) Tissue-specific expression pattern of *TcRtv* in the late last instar larval midgut (A, anterior midgut; M, middle midgut; P, posterior midgut) and C, carcass (whole body minus gut) of *T. castaneum*. *TcRpS6* was used as an internal loading control. (C) Phylogenetic analysis of Rtv orthologs from several orders of insect species was done using MEGA 4.0, with *Ixodes scapularis* included as outlier. Bootstrap analysis of 5000 replications was carried out on trees inferred from the neighbor joining method. (D) Effect of *TcRtv* dsRNA treatment on development of beetles was checked by injecting ~200ng dsRNA into penultimate instar larvae, last instar larvae and pharate pupae (n = 20). All *TcRtv* dsRNA-injected insects died (100% mortality) at each stage of molting. dsRNA for the Vermilion gene (TcVer) was used as a control (n = 20 each). Scale bar = 1 mm. (E) Effect of *TcRtv* dsRNA treatment on the transcript levels. Three to four days after injection, dsRNA-injected insects were collected from each stage of development for RNA extraction and cDNA preparation. RT-PCR was performed (28 cycles) to check the level of *TcRtv* transcripts using gene-specific primers. *TcRpS6* was used as a loading control (24 cycles).

adult molts also failed to shed their old cuticles. Control insects injected with the same dose of dsRNA against the eye pigmentation gene, *TcVer*, developed normally except for the loss of eye color.

### **TcRtv is important for the maintenance of procuticular chitin**

Because of the terminal phenotypic resemblance of insects treated with dsRNAs against *TcRtv* or *TcChs-A*, we suspected that, like RNAi for *TcChsA* (Arakane et al., 2005), dsRNA for *TcRtv* also may lead to a reduction of procuticular chitin content, resulting in loss of mechanical strength of the new cuticle and failure to complete ecdysis. To test this hypothesis, insects at the pharate pupal stage were treated with *TcRtv*-specific dsRNA and their chitin qualitatively and quantitatively probed just prior to the adult molt (pharate adult). Confocal microscopic analysis of the pharate adult elytra stained with a chitin-binding domain (CBD) probe (fluorescent rhodamine-conjugated CBD) showed a near-complete loss of chitin in the new cuticle of *TcRtv*-depleted insects, when compared to control insects (Fig 3.3 A).



**Figure 3.3 TcRtv is important for the sustenance of procuticular chitin.**

(A) Chitin staining (red) of cryosections of pharate adult insects (n=5) containing both the old pupal cuticle (PC) and the newly synthesized elytral cuticle (EC). Nuclei are stained with DAPI (blue). Scale bar, 10  $\mu$ m. (B) Quantitative analysis of chitin content from whole animals at pharate pupal and pharate adult stages was carried out using a modified Morgan-Elson assay (n = 5) and reported as mean  $\pm$  SE. dsRNA for *TcVer* and *TcChs-A* were injected as negative and positive controls, respectively. Statistical significance was computed by the Student's *t*-test. Means identified by different letters are significantly different at  $p < 0.05$ .

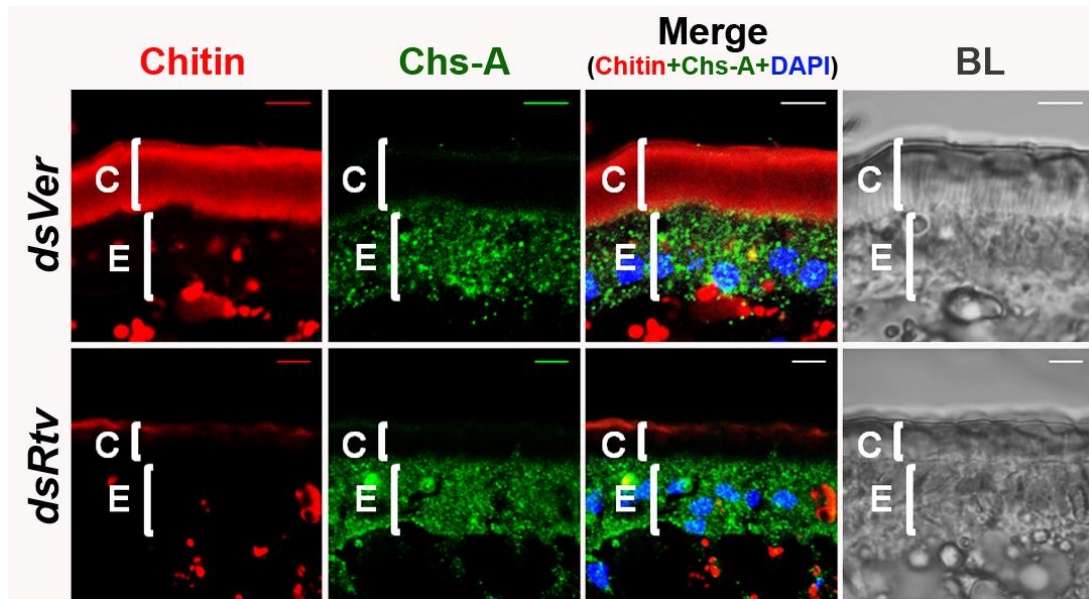


A similar reduction in new cuticular chitin was also detected in the pharate adult insect body wall (Fig 3.5 B, column 1- row 2). Independently, quantitative analysis of total body chitin content by the modified Morgan-Elson assay confirmed chitin depletion in these insects during both larval-pupal and pupal-adult molts (Fig 3.3 B). The reduction of chitin content following RNAi of *TcRtv* was comparable to that observed in *TcChs-A*-depleted insects, and varied depending on the stage of insect development from ~ 2-to 10-fold relative to control insects (Fig 3.3 B). Collectively, these data suggest that TcRtv affects molting by modulating the level of chitin, predominantly in the newly forming cuticle(s).

### **TcRtv prevents chitinase-mediated degradation of procuticular chitin**

The total amount of chitin in an insect is likely to change dynamically during periods of growth as a result of repeated cycles of cuticle deposition and turnover. The dynamics are more complex during molting when there is an overlap of the period of chitin synthesis in the new cuticle with that of chitin degradation in the old cuticle. As a result, several possible mechanisms by which TcRtv may regulate chitin levels in the procuticle can be envisioned. The loss of chitin following RNAi for *TcRtv* suggests that this protein might be an activator of chitin synthesis or an inhibitor of chitin degradation. In the first scenario, TcRtv might affect chitin synthesis via its effect on *TcChs-A* at the transcriptional, post-transcriptional, translational or post-translational levels.

However, our observation that steady-state levels and cellular distribution of TcChs-A protein were similar in control and TcRtv-depleted insects (Fig 3.4) argues against a direct effect of TcRtv on *TcChs-A* expression and localization. An alternative hypothesis that would explain the observed reduction in chitin in the newly forming procuticle following TcRtv RNAi is that TcRtv protects newly synthesized chitin from molting fluid chitinases. Indeed, we have shown recently that chitin is co-localized with chitinases, which are not excluded from the newly forming procuticle as had been assumed in the past. We have further shown that the chitin-binding protein, TcKnk, is needed to protect the nascent chitin in the procuticle from chitinase-mediated degradation (Chaudhari et al., 2011). To test the hypothesis that TcRtv also protects chitin from chitinase mediated degradation, we down-regulated the expression of the major molting fluid chitinase (*TcCht-5*) at the pharate adult stage either alone or in combination with *TcRtv* and subjected these samples to quantitative chitin content analysis. Remarkably, the



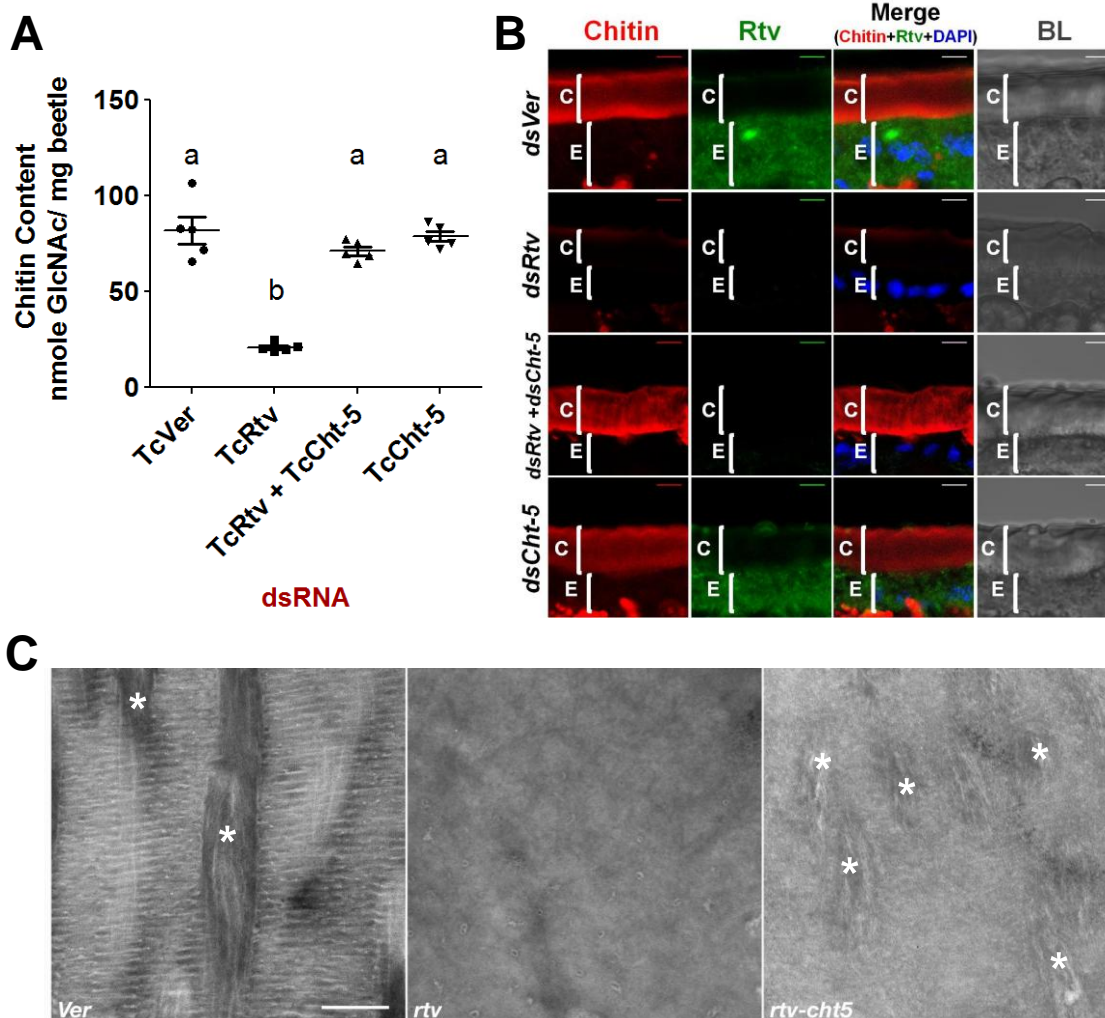
**Figure 3.4 Localization of TcChs-A in insects treated with dsRNA for *TcVer* and *TcRtv*.**

*T. castaneum* pharate adult lateral body wall sections (20  $\mu\text{m}$ ) to analyze the new cuticle were stained with TcChs-A antibody. No visible differences in the cellular distribution of TcChs-A protein localization were detected in absence of TcRtv. Chitin (red); TcChs-A (green); DAPI (blue); C, cuticle; E, epithelial cell. Scale bar = 5  $\mu\text{m}$ .

analysis clearly revealed that insects subjected to RNAi for *TcRtv* alone exhibited significant chitin reduction, but co-depletion of both *TcRtv* and *TcCht-5* transcripts resulted in no apparent loss or reduction of cuticular chitin content relative to control (*TcVer*) at the pharate adult stage (Fig 3.5A). The confocal microscopic data showed a near complete loss of chitin in the new procuticle following depletion of *TcRtv* transcripts compared to the dsRNA *Ver*-treated insects. Following RNAi for both *TcCht-5* and *TcRtv*, rhodamine-CBD staining indicated that the levels of chitin within the new cuticle of body wall were restored to the same level as *dsVer* controls. (Fig 3.5B, compare row 3 with row 1).

### **TcRtv affects the ultrastructure of the procuticle**

To gain a better understanding of the nature of the restored chitin in the procuticle following depletion of transcripts for both *TcCht-5* and *TcRtv*, we performed TEM analysis of the elytra of insects treated with the indicated combination of dsRNA(s). Sections of elytra from control



**Figure 3.5 TcRtv prevents chitinase-mediated degradation of procuticular chitin.**

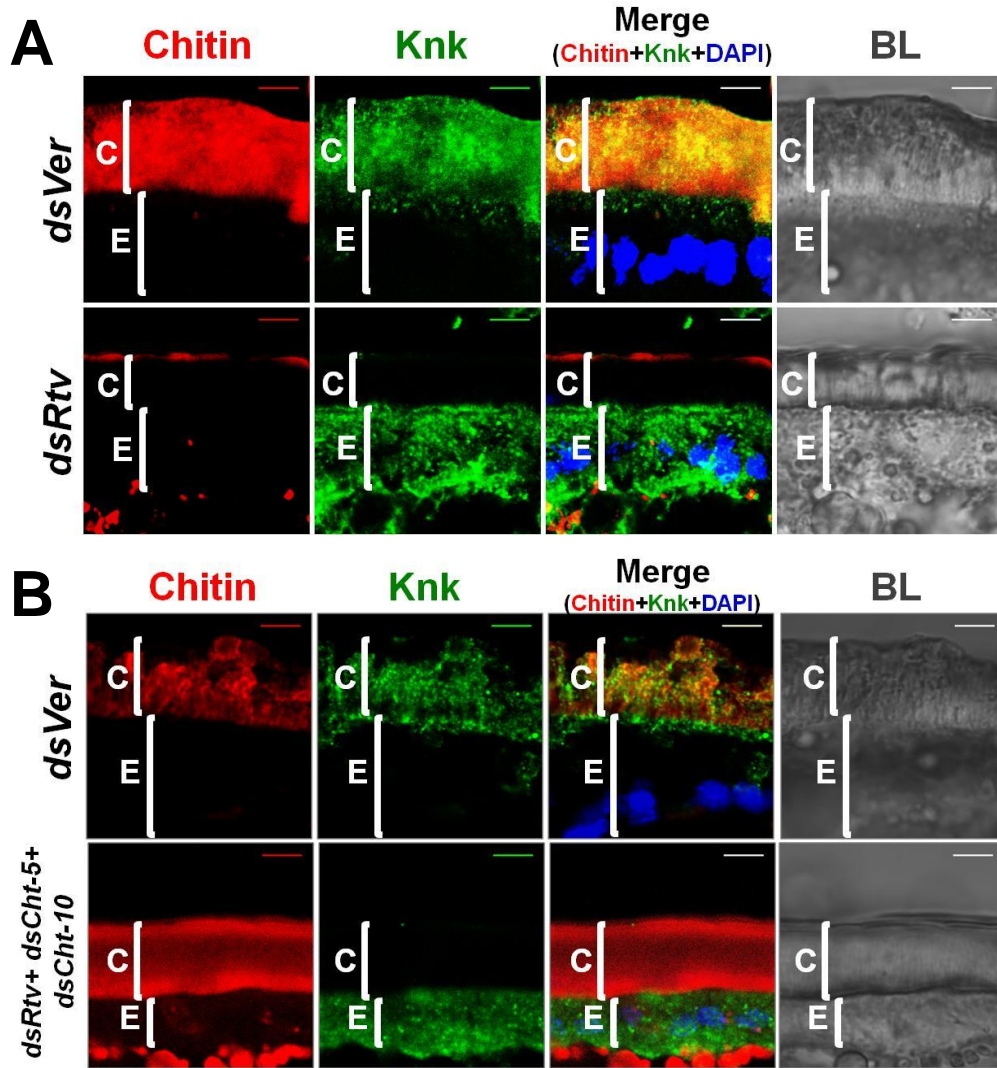
(A) Quantitative analysis of total chitin content of per mg of pharate adult insects after dsRNA treatment for *TcVer*, *TcRtv*, *TcRtv+TcCht-5* and *TcCht-5* was performed using a modified Morgan-Elson assay (n = 5). Chitin depletion resulting from *TcRtv* RNAi was restored to control levels following double RNAi for both *TcRtv* and *TcCht-5*. (B) Immunohistochemical analysis of *TcVer*, *TcRtv*, *TcRtv+TcCht-5* and *TcCht-5* dsRNA-treated insects revealed rescue of the chitin reduction phenotype upon co-knockdown of *TcRtv* and *TcCht-5* (*dsRtv+dsCht-5*). Chitin (red), TcRtv (green), DAPI (blue), C, cuticle; E, epithelial cells. Scale bar = 5  $\mu$ m. (C) Ultrastructure of pharate adult elytral cuticle. Elytra from *Ver* dsRNA-treated animals (*Ver*) consist of horizontal brick-wall like chitin laminae with respect to the apical surface of epithelial cells that are occasionally separated by vertical channels (\*, perpendicular to the apical plasma membrane) that contain a fibrous material, probably chitin. The elytral cuticle of pharate adults treated with dsRNA for *TcRtv* and *TcRtv* along with dsRNA for *TcCht-5* does not restore the laminar organization of chitin. Number of vertical channels (\*) increased in *Rtv+Cht-5* dsRNA treated insect elytral cuticle. Scale bar for *ver*, *rtv* and *rtv-cht5* = 500 nm.

insects were characterized by a well-organized laminar architecture. Along with the drastic reduction in procuticular chitin, the laminar architecture was completely disrupted following RNAi for *TcRtv*. Although simultaneous down-regulation of *TcRtv* and *TcCht-5* transcripts resulted in the restoration of chitin within the procuticle, the laminar organization was not reestablished (Fig 3.5C). Interestingly, this phenotype was indistinguishable from the phenotype that we have recently observed in elytra of pharate adults depleted of transcripts for both *TcKnk* and *TcCht-5*. In addition to the laminar architecture of the cuticle, vertical channels (probably made up of chitin) were seen in elytral cuticle of *TcVer* dsRNA treated insects. Overall number of these vertical channels increased in *TcRtv+TcCht-5* knockout upon complete recovery of the chitin.

### **TcRtv is essential for the procuticular localization of TcKnk**

Since the phenotypes of RNAi for *TcRtv* and *TcKnk* are indistinguishable, we considered the possibility that these two proteins may be involved in the same linear pathway. Immunohistochemistry of sections of control insects (dsVer-treated) and those treated with dsRNAs specific for *TcRtv* and *TcCht-5*, either alone or in combination, was carried out using a polyclonal antibody against Knk. Depletion of *TcRtv* transcripts alone resulted in the mislocalization of TcKnk from its normal distribution predominantly in the procuticle to an exclusive presence within the epidermal cells, perhaps with some TcKnk in the plasma membrane (Fig 3.6A).

More importantly, TcKnk was excluded from the procuticle in the absence of TcRtv. This exclusion of TcKnk from the newly forming procuticle may have been due to either the absence of chitin in the procuticle or the absence of the TcRtv protein. It is conceivable that the observed loss of chitin in the newly forming procuticle in dsRNA TcRtv-treated insects was a secondary consequence of the exclusion of TcKnk, rather than a direct result of TcRtv malfunction. TcKnk has been shown to protect chitin in the procuticle from chitinases, which are co-localized with chitin (Chaudhari et al., 2011). Alternatively, TcRtv may have affected TcKnk's localization via protein-protein interaction. To distinguish between these two possibilities, TcKnk localization was examined in insects depleted of both *TcRtv* and *TcCht-5* transcripts by coinjection of both dsRNAs. This enabled us to disable TcRtv function without concomitant depletion of procuticular chitin, and thus observe the direct effect of TcRtv on localization of TcKnk



**Figure 3.6 TcRtv influences the localization of TcKnk to the procuticle.**

Cryosections of lateral body wall of *T. castaneum* pharate adults (20  $\mu\text{m}$  thick) of control *TcVer* (*dsVer*), *TcRtv* (*dsRtv*) or *TcRtv+TcCht-5+TcCht-10* (*dsRtv+dsCht-5+dsCht-10*) dsRNA-treated insects were immunostained with *D. melanogaster* Knk (DmKnk) antiserum (green). (A) Mislocalization of TcKnk following RNAi for *TcRtv*. (B) Mislocalization of TcKnk in pharate adults co-injected with a mixture of dsRNAs for *TcRtv*, *TcCht-5* and *TcCht-10*. Mislocalization of TcKnk in the absence of TcRtv, even in presence of chitin, indicates importance of TcRtv in proper localization of TcKnk.

independently of any possible contributing effects of procuticular chitin itself. Although chitin levels remained at near-normal levels like control *TcVer* in the procuticle after such treatment, TcKnk was mislocalized, remaining entirely inside the cell rather than being secreted into the procuticle (Fig 3.6B). The distribution of TcKnk in these double-knockouts was indistinguishable from that observed in insects treated with dsRNA for *TcRtv* alone. These observations support the hypothesis that TcRtv directly facilitates the trafficking of TcKnk into the procuticle.

## Discussion

The assembly of chitin into the laminated procuticle of insects is a complex process that involves the coordinated action of multiple proteins involved in the synthesis and modification of chitin as well as other chitin-binding and chitin-organizing proteins. While the functions of chitin synthases, deacetylases and chitinases have been studied in detail (Arakane et al., 2009; Arakane et al., 2005; Zhu et al., 2008a; Zhu et al., 2008b), the role(s) of chitin-binding/organizing proteins are less well understood (Chaudhari et al., 2011; Jasrapuria et al., 2010). Mutational analysis in *D. melanogaster* has implicated two genes, *TcKnk* and *TcRtv*, in the organization of tracheal tube chitin and of the embryonic cuticular matrix (Moussian et al., 2005; Moussian et al., 2006b). More recently, we have shown that the TcKnk protein is localized primarily in the procuticle, co-occurring with chitin (Chaudhari et al., 2011). Furthermore, we have shown that procuticular TcKnk is indispensable in protecting chitin from the action of chitinases, which are not excluded from chitin in the newly forming procuticle as previously believed. In addition, TcKnk is needed for the organization of procuticular chitin into an ordered laminar array. However, the role of the protein TcRtv has not been studied extensively even though the phenotypes of *Rtv* and *Knk* mutants of *D. melanogaster* are virtually indistinguishable (Moussian et al., 2005; Moussian et al., 2006b).

### A role for TcRtv in new cuticle maintenance during molting

We have studied the role of TcRtv in greater detail using larvae and pupae of *T. castaneum*, which have been used previously in RNAi studies for elucidating the function of various genes involved in chitin synthesis and organization (Arakane et al., 2005; Chaudhari et al., 2011; Zhu et al., 2008b). As in the case of *D. melanogaster*, a single gene encoding an Rtv-like protein is present in the *T. castaneum* genome as well as in several other insect genomes that have been

completely sequenced. Interestingly, a single *Rtv* ortholog is also present even in non-insect arthropod species including the water flea, *Daphnia pulex* and the deer tick, *Ixodes scapularis*, suggesting that *Rtv* may not be limited to the insecta and may have a conserved biological function in other species that produce a chitinous exoskeleton. On the other hand, we could not identify *Rtv* homologs in the genome of the nematode, *Caenorhabditis elegans*, which does synthesize chitin in its eggshells and the pharyngeal luminal walls, but not in its exoskeleton.

Expression of the *TcRtv* gene throughout all stages of insect development when cuticle is being deposited and the higher levels of transcripts for this gene during periods of rapid tissue remodeling (e.g. the pharate pupal and pharate adult stages) are consistent with a role in cuticulogenesis. The expression of *TcRtv* roughly parallels that of another gene, *TcKnk*, which is involved in the organization of chitin bundles in the tracheal lumen and in the epidermal cuticle (Chaudhari et al., 2011; Moussian et al., 2005). Even though both of these genes are predicted to encode proteins with GPI anchors by the program big-PI Predictor, PredGPI and GPI-SOM, only *TcKnk* is released from the plasma membrane of cells expressing this protein by treatment with PI-PLC, an enzyme known to cleave the GPI anchor of membrane-bound proteins (Chaudhari et al., 2011; Moussian et al., 2006b), whereas *TcRtv* is not released by this treatment. In agreement with these observations, *TcKnk* is found to be predominantly located in the procuticle, presumably as a result of the action of a PI-PLC (Chaudhari et al., 2011; Moussian et al., 2005), whereas *TcRtv* is found predominantly inside the cell. We are unsure whether *TcRtv* has a GPI anchor or merely has a membrane-spanning segment at the C-terminus. We did not find any evidence for the presence of *TcRtv* protein in the procuticle. Therefore, we predict that *TcRtv* cannot have a direct role in protecting chitin from chitinases and that the loss of procuticular chitin observed after RNAi of *TcRtv* expression must be via an indirect effect. The finding that there are no significant differences in the amount or localization of *TcChs-A* in control and dsRNA *TcRtv*-treated insects also suggests that *TcRtv* does not have a direct effect on *TcChs-A* expression or localization. Finally, the full recovery of chitin levels in the procuticle in the absence of *TcRtv* upon down-regulation of chitinases rules out any role for *TcRtv* in the synthesis and transport of chitin to the procuticle. Unfortunately, we have not been able to carry out binding studies using chitin and *TcRtv* due to our inability to obtain the latter in a homogeneous form from insect cell cultures infected with recombinant baculoviruses containing this gene.

In the absence of any evidence for a direct interaction between chitin and TcRtv protein, we have sought to explain the observed phenotypes following RNAi for *TcRtv* in terms of the changes in the amount and localization of another cuticle-associated protein, TcKnk, which is known to have affinity for chitin and is predominantly located in the procuticle. The phenotypes of *T. castaneum* subjected to RNAi for *TcRtv* or *TcKnk* are virtually indistinguishable, and include the loss of procuticle-associated chitin and loss of the laminar organization of the procuticle. Our immunolocalization studies did reveal that after RNAi for *TcRtv*, TcKnk protein is mislocalized inside the cell instead of in its normal location in the procuticle. This situation is reminiscent of our earlier observations that after RNAi for *TcChs-A* or treatment with the chitin synthesis inhibitor, nikkomycin, TcKnk is similarly mislocalized inside the cell, leading to a loss of procuticular chitin as a result of a failure to protect procuticular chitin from degradation by molting-associated chitinases (Chaudhari et al., 2011). Thus, all of the observed phenotypic changes following RNAi for *TcRtv* can be accounted for in terms of its effect on the localization of TcKnk protein.

### **How does TcRtv protein affect the movement of TcKnk to the procuticle?**

We have demonstrated that the absence of chitin in the procuticle alone does not account for the mislocalization of the TcKnk protein in the absence of TcRtv, because simultaneous RNAi of chitinase genes, while restoring procuticular chitin, fails to rescue the intracellular mislocalization of TcKnk. It appears that the targeting of TcKnk protein to the procuticle requires the presence of TcRtv. Whether this is due to a direct protein-protein interaction between TcRtv and TcKnk or through a more complex pathway remains to be established.

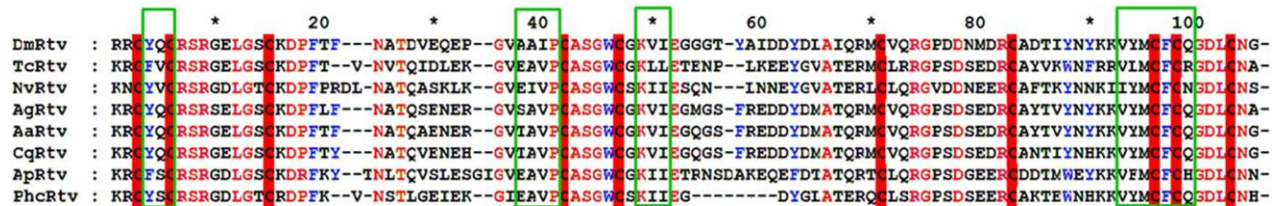
Mislocalization of TcKnk has been previously reported in tracheal tube expansion mutants of *D. melanogaster* homozygous for alleles of genes encoding five proteins that are components of septate junctions, namely *Fas2*, *sinuous*, *mega*, *bulbous* and *ATP  $\alpha$*  (Moussian et al., 2006b). However, unlike our results with epidermal cells of the body wall of *T. castaneum*, mislocalization of Knk in the tracheal luminal cells of *Rtv* and *Chs-A* mutants was not observed by the above workers in *D. melanogaster*. Additional experiments are needed to clarify whether the mislocalization is restricted only to specific tissues such as the elytral and body wall epidermal cells that we have focused on. At any rate, it is clear that movement of Knk to its



apical plasma membrane location or as demonstrated here to the extracellular matrix is dependent on several proteins including Rtv.

### TcRtv may be a member of the Ly-6 superfamily of proteins

The Ly-6 protein superfamily is composed of several membrane-bound receptors, GPI-anchored proteins and soluble ligands. These proteins are characterized by the presence of an approximately 100-residue long extracellular motif called the three-finger domain (TFD) (Hijazi et al., 2009). In the prototypical structure described for proteins of this family (the sea snake venom neurotoxin, erabutoxin b), the cysteines form a series of disulfide bridges that stabilize the protein core and allow three finger-like loops to protrude (Bamezai, 2004; Hijazi et al., 2009). These finger-like extensions with variable sequences are believed to enable interaction of these proteins with a broad range of substrates, each with a high degree of specificity. Besides the TFD, Ly-6 proteins also contain an N-terminal signal peptide that ensures their transport to the extracellular surface through the endoplasmic reticulum and via secretory vesicles, as well as a C-terminal hydrophobic domain that allows the addition of a GPI anchor (Hijazi et al., 2009). Having arrived at the plasma membrane surface, these proteins may be secreted to the extracellular milieu and function as ligands, or may stay attached to the membrane by the GPI



**Figure 3.7 TcRtv may be a Ly-6 family protein.**

Alignment of Rtv proteins from different insect species shows 10 conserved cysteines (solid red boxes) and aromatic residues (blue), the latter which are predicted to bind with chitin. Four predicted  $\beta$ -strands are indicated by green boxes.

anchor. Members of the Ly-6 protein superfamily are present in all metazoan genomes and have been implicated in a wide variety of physiological processes ranging from remodeling of the extracellular skeletal muscle matrix in higher eukaryotes to tracheal tube morphogenesis and

septate junction (SJ) formation in insects (Bamezai, 2004; Hijazi et al., 2009). A search of the SCOP database revealed that the *T. castaneum* retroactive (TcRtv) protein shares broad structural similarity with the snake-toxin like or TFD family of proteins (Moussian et al., 2005). TcRtv contains 11 cysteines of which 10 are predicted to adopt the TFD fold. Also present within TcRtv is a conserved asparagine that is immediately adjacent to the last canonical cysteine of this fold. These characteristics, along with the N-terminal signal peptide and C-terminal GPI anchor (additional domains often found linked to Ly-6 protein family), suggest that TcRtv may be a member of the Ly-6 family of proteins (Fig 3.7). The observations that TcRtv and DmRtv are not released from cells suggest that they may function similarly to the septate junction proteins and facilitate the movement of Knk protein. However, unlike the septate junction proteins of *D. melanogaster*, both TcRtv and DmRtv promote transport of Knk to the apical plasma membrane and possibly its release as well. This conclusion is based on the observation that in the absence of TcRtv, TcKnk protein remains inside the cell, presumably in vesicles. Future experiments are required to precisely identify the intracellular location of Knk and the mechanism by which Rtv influences the transport of Knk to the apical side of the epidermal cell.

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**Chapter 4 - Chitin synthase A, Knickkopf and Retroactive are proteins required for embryonic serosal cuticle formation in the red flour beetle, *Tribolium castaneum***

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## Abstract

Chitin, a homopolymer of  $\beta$ -1-4-linked N-acetylglucosamine synthesized by chitin synthase A (Chs-A), is organized in the procuticle to give stability and integrity to the underlying insect epidermal cells and other tissues. Our previous work has revealed an important role for two proteins from *Tribolium castaneum* (the red flour beetle) named Knickkopf (TcKnk) and Retroactive (TcRtv) in postembryonic cuticular chitin maintenance. TcKnk and TcRtv were shown to be required for protection and organization of newly synthesized procuticular chitin. To study the functions of *TcKnk* and *TcRtv* in embryonic cuticle formation in *T. castaneum*, dsRNAs specific for these two genes were injected into two week-old adult females. The effects of dsRNA treatment on oviposition, egg hatching and adult survival were determined. Insects treated with dsRNA for *TcChs-A* and *TcVer* were used as controls for these experiments. Like *TcChs-A*, *TcKnk* dsRNA-treated adult females laid normal (similar to control *TcVer* RNAi) numbers of eggs, but the eggs did not hatch. Although *TcRtv* dsRNA treatment also resulted in a normal oviposition rate in comparison with control insects subjected to *TcVer* RNAi, approximately 90% of those eggs did not hatch. The remaining 10% of the eggs hatched into first instar larvae that died without molting to the second instar. Chitin content analysis following *TcKnk* and *TcRtv* parental RNAi revealed approximately a 50% reduction in chitin content in eggs in comparison with control *TcVer* RNAi, whereas *TcChs-A* dsRNA-treatment led to >90% loss of chitin. Furthermore, transmission electron microscopic (TEM) analysis of serosal cuticle from *TcChs-A*, *TcKnk* and *TcRtv* dsRNA-treated insects revealed a complete absence of laminar organization of procuticle in comparison with *TcVer* dsRNA-treated controls, which exhibited normal laminar organization of procuticular chitin. The results of this study demonstrate that in addition to their essential roles in maintenance and organization of chitin in epidermal cuticle in larval and later stages of insect development, TcKnk and TcRtv also are required for serosal cuticle chitin synthesis and organization during embryonic development of *T. castaneum*.

## Introduction

Cuticle or exoskeleton is the primary barrier that protects insects from mechanical or biological damages (Moussian, 2010). The insect cuticle is typically divided into three major layers, namely the outermost waxy envelope layer, the middle protein-rich epicuticle, and the innermost chitinous, protein-rich procuticle (Locke, 2001). Chitin, a homopolymer of  $\beta$ -(1,4)-linked N-acetylglucosamine, is the major component of procuticle in many insect species (Merzendorfer, 2006; Muthukrishnan, 2012). Chitin is synthesized by a membrane-bound chitin synthase-A, and interacts with various assortments of cuticular proteins in different anatomical parts of the insect to make the corresponding cuticles rigid or flexible as needed (Merzendorfer, 2006; Willis, 2010). RNA interference (RNAi) experiments in the red flour beetle, *Tribolium castaneum*, have revealed an essential role for *chitin synthase-A* (*TcChs-A*) in synthesis of cuticular chitin (Arakane et al., 2005b; Arakane et al., 2008). Depletion of *TcChs-A* transcripts by dsRNA treatment leads to hatching failure at the embryonic stage and molting failure at post-embryonic stages of development (larval-larval, larval-pupal and pupal-adult molts) (Arakane et al., 2005b; Arakane et al., 2008). Our previous work has revealed the important roles for two other *T. castaneum* genes, *Knickkopf* (*TcKnk*) and *retroactive* (*TcRtv*), in the maintenance of procuticular chitin levels in pharate pupal and pharate adult stages. Administration of dsRNA for *TcKnk* or *TcRtv* disrupted all types of molts as well as reduced total chitin levels to an extent comparable to that observed after down-regulation of *Chs-A* transcripts (Chaudhari et al., 2011) (see Chapter 3). *TcRtv* has been shown to be important for proper trafficking of *TcKnk* protein from inside epidermal cells to the procuticular chitin laminae, where *TcKnk* colocalizes with chitin and protects it from degradation by chitinases. Whether this protection is a direct effect or an indirect effect of *Knk* involving additional proteins is currently unknown. But it is clear that *Chs-A*, *Knk* and *Rtv* cooperate to maintain the levels of chitin in the cuticle produced by cells of ectodermal origin.

While the epidermal cells (and other epithelial cells of ectodermal origin in tissues such as hindgut, crop and tracheae) are largely responsible for the secretion of cuticles associated with the insect exoskeleton in different anatomical locations (head capsule, body wall, elytron, wing), in developing embryos there is another source of a cuticular tissue. In most winged insects, the extra-embryonic epithelium, the serosa, secretes a cuticle that lies between the endochorion and the single layer of serosal cells. In *T. castaneum*, the serosal tissue completely covers the



developing embryonic and amniotic primordia at the time of dorsal closure of about 12 h post-oviposition (Handel et al., 2000). In *Aedes aegypti* secretion of the white serosal cuticle occurs between 11 and 13 h after oviposition at the time of complete germ band extension (Rezende et al., 2008). The appearance of this serosal cuticle was shown to be correlated with the acquisition of desiccation tolerance. Furthermore, using a variety of techniques, this serosal cuticle was found to contain chitin, a normal component of insect cuticle secreted by epidermal cells. At later stages of embryogenesis, the developing tracheal system, and much later, epidermal cells secrete chitinous cuticles. It was of interest to compare the identities and functions of genes involved in serosal cuticle synthesis, secretion and organization with those involved in the synthesis of epidermal cuticle. In this study, we report the requirements for *TcChs-A*, *TcKnk* and *TcRtv* genes for serosal cuticle formation in early stages of *T. castaneum* embryonic development using parental RNAi techniques to down-regulate transcript levels for these genes.

## Material and methods

### Insect cultures

The *T. castaneum* GA-1 strain was used for all experiments. Insects were reared at 30°C in wheat flour containing 5% brewer's yeast under standard conditions as described previously (Beeman and Stuart, 1990).

### RNA interference studies

dsRNAs for *TcKnk*, *TcRtv*, *TcChs-A* and *TcVer* were synthesized and injected into 2 week-old mature female adults (n = 20). One day after injections, female were mated with an equal number of adult males, and eggs were collected at four-day intervals. Gene-specific forward and reverse primer pairs with additional T7 promoter sequences at the 5'-ends were synthesized (Table 4.1) and used for the preparation of dsRNAs utilizing an Ampliscribe T7-Flash Transcription Kit (Epicentre Technologies) on indicated cDNA templates as described previously (Arakane et al., 2005a). dsRNA for *T. castaneum Vermilion* (*TcVer*), a gene required for eye pigmentation, was used as a control for monitoring RNAi effectiveness, whereas dsRNA for *chitin synthase-A* (*TcChs-A*) was used as a positive control for chitin depletion. Total RNA extraction, first-strand cDNA synthesis and PCRs were carried out as described previously (Arakane et al., 2005a).

**Table 4.1 Primer sequences for generation of dsRNAs.**

<b>dsRNA</b>	<b>Sequence (5'-3')</b>	<b>Nucleotide positions</b>	<b>dsRNA length, bp</b>
<i>TcKnk</i>	F= TAATACGACTCACTATAGGGTTGGGACAATATAAAGGTG R= TAATACGACTCACTATAGGGTAAATTGAGCCCTCGG	1036-1201	165
<i>TcRtv</i>	F= TAATACGACTCACTATAGGGTGGAAATTCTCAAACGC R= TAATACGACTCACTATAGGGTAGAGACAGAAAATCC	72-233	161

### **Dissection of ovaries and testes**

Ovaries were dissected from female adults (2 week-old) in phosphate buffer saline using #5 forceps. Images were taken on a Lieca microscope at 1.25X zoom.

### **Chitin content analysis of eggs**

Pools of ~200eggs from each group of dsRNA-treated insects were collected every 4 d and stored at -80°C. Total chitin content analysis of 4-d-old eggs was performed using a modified Morgan–Elson method as described previously (Arakane et al., 2005b).

### **Transmission electron microscopic analysis (TEM)**

For TEM analysis, gene-specific dsRNAs for *TcKnk*, *TcRtv*, *TcChs-A* or *TcVer* were injected into mature adults (2-week-old). Mature embryos (4-d-old) were collected, fixed and processed for TEM analysis as described earlier (Moussian et al., 2005).

## Results and Discussion

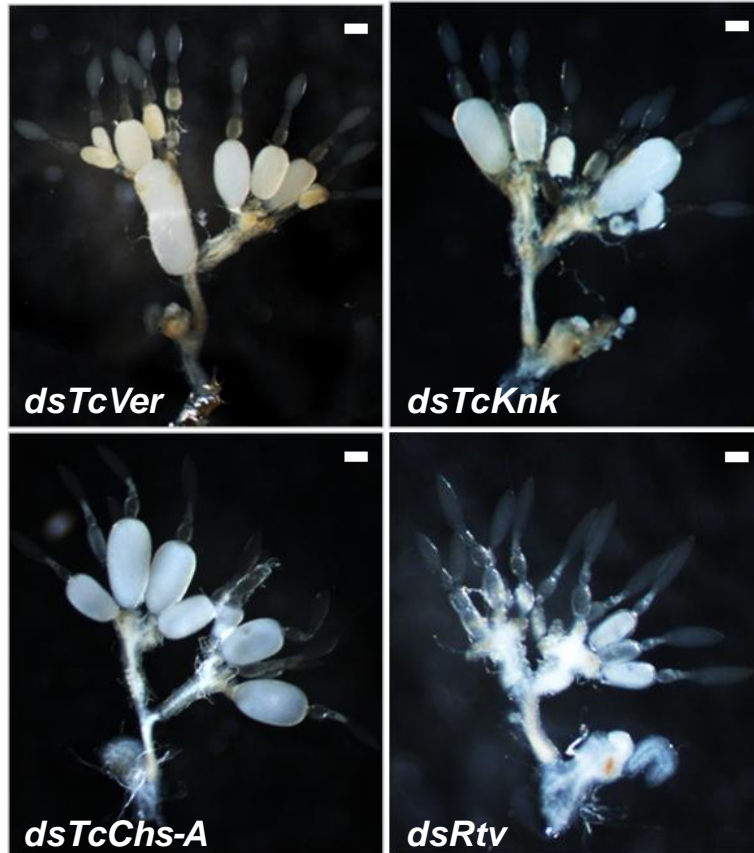
We have previously reported about the roles of *TcChsA*, *TcKnk* and *TcRtv* in the formation and assembly of chitin in post-embryonic cuticles of epidermal origin (Arakane et al., 2005b; Arakane et al., 2008; Chaudhari et al., 2011). Unlike post-embryonic stages, during embryogenesis there are three different types of cuticles produced. First, the serosa secretes a thin cuticle that surrounds the entire amniotic and developing embryonic rudiments. Midway through embryogenesis, tracheal lining cells, and later, the larval epidermis secrete cuticles. To study the roles, if any, of the *TcChsA*, *TcKnk* and *TcRtv* genes on the formation and architecture of the cuticle during embryogenesis, we injected dsRNA specific for each of these three genes separately into fertile adult females, and after one day mated them with control males. Eggs from these crosses were collected periodically, counted and incubated under standard conditions and allowed to hatch. Depletion of *TcChs-A* or *TcKnk* transcripts in adult female beetles had no effect on adult viability, fecundity or oviposition, but none of the embryos hatched (Table 4.2).

**Table 4.2 Effect of parental RNAi on oviposition and egg hatching in *T. castaneum*.**

dsRNA	Number of eggs laid by injected adult females*	% Egg hatching	Adult parental female survival (n=20)
TcVer (Control)	160	100	20
TcChs-A	137	0	20
TcKnk	155	0	20
TcRtv	148	10	20

\*the number of eggs is from 20 adult female beetles collected over a four day period.

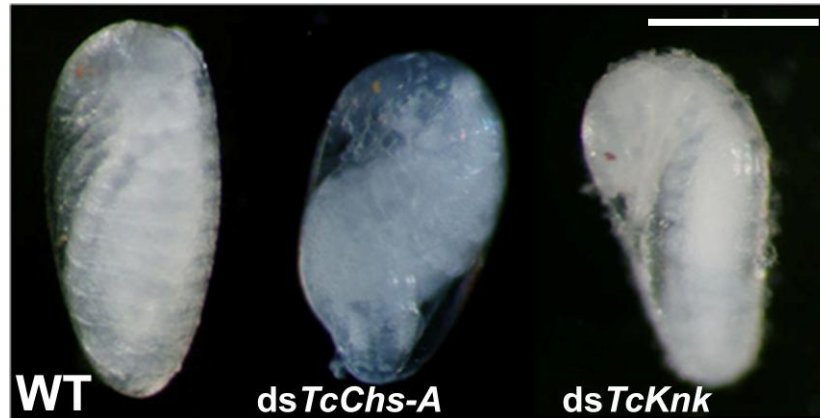
As in the case of *TcChs-A* or *TcKnk* dsRNAs, *TcRtv* dsRNA treatment had no effect on adult survival or oviposition, but ~90% of developing embryos failed to hatch. The remaining ~10% of the eggs after parental RNAi for *TcRtv* hatched, but the hatchlings were unable to molt. Microscopic analysis of dissected ovaries of injected adult females indicated that after oviposition embryonic development proceeded normally (Fig 4.1).



**Figure 4.1 Effect of *TcChs-A*, *TcKnk* and *TcRtv* dsRNA-treatment on ovarian development.** Ovaries were dissected out from two-week-old adult females after *TcChs-A*-, *TcKnk*- or *TcRtv*-specific dsRNA treatment. None of the dsRNA treatments had any discernible effect on oocyte development. Scale bar= 1 mm.

Mature embryos had normal body segmentation and fully pigmented eyes, but were somewhat bloated and twisted, similar to those observed after parental RNAi for *TcChs-A* (Fig 4.2, *TcRtv* RNAi data not shown) (Arakane et al., 2008). Reduction in chitin content has been shown to result in a weakened cuticle leading to enlargement of the embryo (a blimp-like phenotype) in *Drosophila melanogaster* and failure of the eggs to hatch in *Chs-A* mutants (Ostrowski et al., 2002).

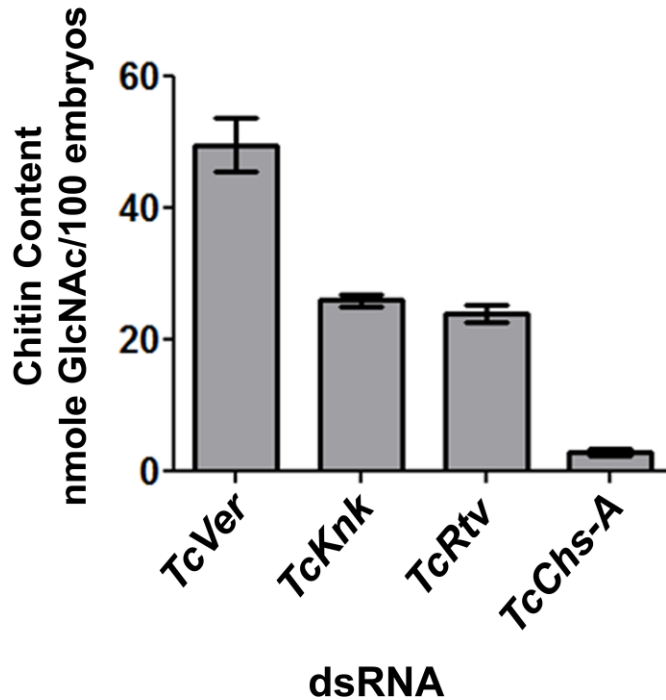
Because of the phenotypic similarity of embryos collected from *TcChs-A*, *TcKnk* or *TcRtv* dsRNA-treated adult female beetles, we hypothesized that changes in chitin content may



**Figure 4.2 Effect of dsRNA-treatment for *TcChs-A* and *TcKnk* into adult females of *T. castaneum* on egg hatch.**

Approximately 200 ng dsRNA per insect was injected into 2 week-old adult females (n = 20), followed by mating with an equal number of untreated males. Adult females injected with dsRNA for *Tribolium Vermilion* (*dsTcVer*) were used as a control. Embryos from the *TcChs-A* and *TcKnk* dsRNA treatment had a twisted and enlarged blimp-like phenotype and died without hatching. Scale bar= 1 mm.

account for hatching defects in all three cases. To test this hypothesis, we determined total chitin content of eggs collected from *TcKnk* or *TcRtv* dsRNA-treated adult females by the modified Morgan-Elson assay and compared these values with those determined in eggs from females after RNAi for *TcChs-A*. There was a >90% reduction in chitin content following RNAi for *TcChs-A* and an approximately 50% reduction following RNAi for either *TcKnk* or *TcRtv* (Fig 4.3). These results indicated that all three genes were somehow involved in the pathway of chitin metabolism. Results from our previous work had shown significant reduction in chitin content of whole insects at pharate pupal and pharate adult stages of development in the absence of *TcKnk* or *TcRtv* (Chaudhari et al., 2011). While the role of *Chs-A* is in synthesis and extrusion of chitin fibers (Moussian et al., 2005), *Knk* has a role in the post-synthetic phase, protecting chitin in the procuticle from the hydrolytic activity of molting-associated chitinase(s), which are not excluded from the newly synthesized procuticle. *Knk* also helps to organize the chitin bundles into laminae (Chaudhari et al., 2011; Moussian et al., 2006). It is unclear whether the higher level of organization of chitin bundles itself or the physical masking of chitin by *Knk* is responsible for the resistance of procuticular chitin to chitinases.



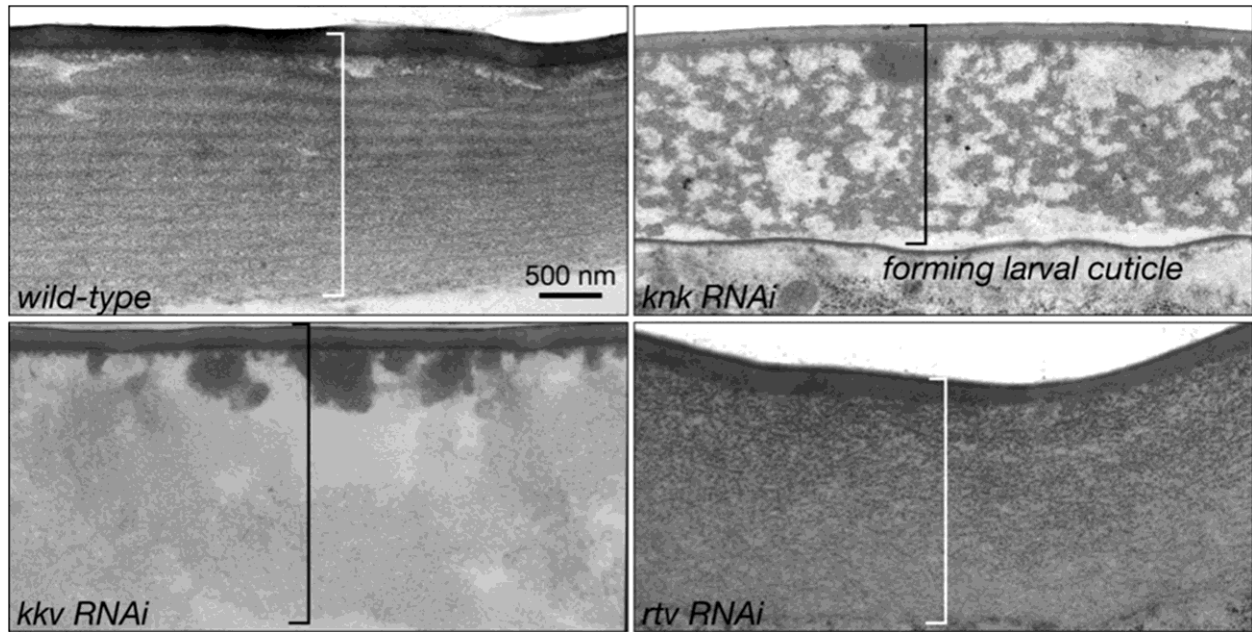
**Figure 4.3 Effect of parental RNAi on *T. castaneum* embryonic chitin content.**

Three batches of four-day-old mature eggs from 20 female beetles injected with *TcChs-A*, *TcKnk* and *TcRtv* dsRNA were collected. Chitin content analysis was carried out using a modified Morgan–Elson method (Arakane et al., 2005b). dsRNA for *T. castaneum* *Vermilion* gene (*dsTcVer*) was injected as a control. GlcNAc= N-acetylglucosamine.

In contrast to pharate pupal and pharate adult stages in which RNAi for *TcKnk* or *TcRtv* results in near-total loss of chitin, the reduction in chitin in the embryo is only about 50%, suggesting that chitinase(s) are less effective in degrading chitin in the developing embryo. One likely possibility is that the partial degradation of chitin during embryonic development is due to low levels or absence of chitinase(s) in embryos. Indeed, the expression profiles of different chitinases during *T. castaneum* development have revealed the presence of transcripts for both *chitinase-5* (*TcCht-5*) and *chitinase-10* (*TcCht-10*) in all post-embryonic stages of development with no or very low expression in embryos (Zhu et al., 2008b). The major chitinolytic activity of molting fluid is due to the group I enzyme, *TcCht-5*, with *TcCht-10* playing a minor secondary role (Chaudhari et al., 2011). *TcCht-5*, highly expressed in the pharate adult stage, is essentially undetectable in the embryonic stage. Furthermore, while RNAi for *TcCht-5* does not affect embryonic development or egg-hatch, RNAi for *TcCht-10* does prevent egg-hatch, indicating

that this enzyme has a critical function during embryogenesis. Whether this enzyme is involved in removal of serosal cuticle or has a role later in embryogenesis (for example removal of tracheal core chitin) is unknown. Nevertheless, it is possible that the activity of TcCht-10, which has five chitinase-like domains with two of them predicted to be catalytically inactive (Zhu et al., 2008a; Zhu et al., 2008b), is much less efficient in degrading chitin than TcCht-5, consistent with only a partial loss of chitin following RNAi for *Knk* and *Rtv* in the embryonic stage.

Chs-A has been shown to be involved in the synthesis of both serosal and larval cuticles during embryonic development in *Aedes aegypti* (Rezende et al., 2008). Interestingly, the production of chitin by the serosal cells is carried out by a specific splice variant, namely *Chs-1a*, whereas both splice variants, *Chs1-a* and *Chs1-b*, are expressed in later stages of embryogenesis when larval cuticle is being deposited. To analyze whether *Knk* and *Rtv* are involved in the synthesis of both types of cuticles in *T. castaneum*, we performed transmission electron microscopic (TEM) analysis of 4-d-old embryos after *TcKnk* and *TcRtv* dsRNA treatment. TEM analysis of control *TcVer* dsRNA-treated embryos revealed proper laminar organization of chitin in the serosal procuticle, whereas *TcChs-A* RNAi embryos were devoid of laminae in the serosal procuticle, presumably because of the near-complete loss of chitin. Instead, clumps of electron dense material could be seen within the procuticle, while the envelope layer appeared to be intact. In comparison with the control, the serosal cuticles of *TcKnk* and *TcRtv* dsRNA-treated embryos with only partial chitin depletion still exhibited a complete loss of laminar organization (Fig. 4.4). The underlying larval cuticle was also devoid of laminae even though an intact envelope could be discerned in embryos following RNAi for *TcKnk* and *TcRtv*. These results are similar to the situation in pharate adult elytra, which also lacked chitin laminae even when the loss of chitin brought about by RNAi for *Knk* was reversed by simultaneous knock-down of chitinase(s) (Chaudhari et al., 2011). The similarity of phenotypes of embryos following RNAi for *Knk* and *Rtv* is consistent with our previous finding that *Rtv* exerts its effects by influencing the localization of *Knk* to the procuticle (see Chapter 3). We have not investigated whether *Knk* causes a similar mislocalization in the serosal cells/cuticle as well, but one would predict that this will be the case.



**Figure 4.4 Transmission electron microscopic (TEM) analysis of serosal cuticle.**

TEM analysis of serosal cuticle after *TcChs-A*, *TcKnk*, *TcRtv*, and *TcVer* dsRNA injections into *T. castaneum* adult females (2 week-old). Mature embryos (4 d-old) were analyzed by TEM. Embryos collected from *TcVer* dsRNA-treatment were used as a control. *TcVer* dsRNA-treated controls showed normal laminar organization of the cuticle, whereas *TcChs-A*, *TcKnk* and *TcRtv* exhibited a loss of laminar organization. Scale bar for all sections = 500 nm.

Our previous work had revealed important roles for *TcKnk* in protecting and organizing chitin into procuticular laminae during post-embryonic stages. *Rtv* acts upstream of *Knk* by targeting the latter protein to the extracellular space (Chaudhari et al., 2011) (see Chapter 3). The results of the present study support the hypothesis that the chitin-protecting and chitin-organizing roles of *Knk* extend to different types of cuticle-forming tissues and that *Rtv* and *Knk* interact in an unknown fashion in tissues other than epidermal tissue, namely the serosal tissue. The similarity in the phenotypes of tracheal tubules in *Knk* and *Rtv* mutants of *D. melanogaster* also suggests a similar interaction between *Knk* and *Rtv* in tracheae (Moussian et al., 2006). It will be interesting to determine whether the spatial organization of the tracheal chitin cylinders follows a similar path even though chitin in these structures is organized into coaxial bundles rather than the laminar arrangement present in epidermal cuticle.



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**Chapter 5 - Characterization and functional analysis of two *Knk*-like genes in the red flour beetle, *Tribolium castaneum***

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## Abstract

Functional analysis of Knickkopf (TcKnk) protein from the red flour beetle, *Tribolium castaneum*, indicates its novel role in organization and protection of the newly synthesized chitin from degradation by chitinases. A bioinformatics search to identify *TcKnk*-like genes in *T. castaneum* genome resulted in the identification of two genes, which we have named *TcKnk2* and *TcKnk3*. Three alternatively spliced variants for *TcKnk3* (*TcKnk3-FL*, *TcKnk3-5'* and *TcKnk3-3'*) have been identified by 5'- and 3'-RACE. RT-PCR analysis revealed that the *TcKnk*-like genes differ in their developmental and tissue-specific patterns of expression. RNA interference (RNAi) for *TcKnk2* and *TcKnk3-3'* transcripts resulted in specific molting arrest at pharate adult stage indicating important roles for these transcripts during post-embryonic development of the beetle. Northern blot analysis of transcripts of *TcKnk3* showed up-regulation of *TcKnk3-3'* transcript upon dsRNA-mediated down-regulation of *TcKnk3-FL* and *TcKnk3-5'* transcripts. Additionally, embryonic development analysis using parental RNAi demonstrated an essential role for *TcKnk2* in oocyte development in the ovaries. Analysis of chitin content of *TcKnk2* and *TcKnk3* dsRNA-injected insects indicated significant decrease in chitin levels at pharate adult stage of development. Ultra-structural studies of *TcKnk2* and *TcKnk3* dsRNA-treated pharate adult insects body wall cuticle and tracheae showed changes in the procuticle integrity of body wall denticles and accumulation of electron dense material at the tips of the tracheal taenidia in comparison with controls. Conservation of all three *Knk*-like genes in all insect species implies important role(s) for TcKnk-like proteins in insect development.

## Introduction

Chitin, a homopolymer of  $\beta$ -1,4 linked N-acetyl glucosamine units, is the major component of insect procuticle (Arakane et al., 2005b). Chitin in the procuticle is synthesized by the integral membrane protein, chitin synthase-A (Chs-A) and is deposited outside of the cell in the form of fiber bundles (Merzendorfer, 2006; Merzendorfer and Zimoch, 2003). Such bundles are arranged in sheets, in which the chitin fiber bundles are combined with cuticular proteins in an interpenetrating network (Locke, 2001; Vincent and Wegst, 2004). Successive layers of chitin sheets are deposited in such a way that the horizontal axes of adjacent laminae follow a helical path (Locke, 2001; Moussian, 2010; Vincent and Wegst, 2004). The role, if any, of cuticular and membrane proteins in organizing chitin laminae into such a helicoidal bundle is unclear.

In addition to cuticle growth, during development insects have to undergo a process of molting, wherein old cuticle is replaced by a new one. Chitinases present in the molting fluid degrade chitin from the old cuticle, providing substrate for new cuticle synthesis. (Locke and Huie, 1979; Noblenesbitt, 1963; Reynolds and Samuels, 1996). A recent study with the red flour beetle, *Tribolium castaneum*, has shown co-localization of chitinases (Chitinase-5) with chitin in the newly synthesized procuticle (Chaudhari et al., 2011). The presence of a chitin-binding protein, Knickkopf (Knk), in the developing new procuticle is essential for protecting chitin from degradation by active chitinases (Chaudhari et al., 2011). Although the exact mechanism of chitin protection is still unclear, Knk's ability to bind with chitin was predicted to lead to a masking effect that prevents degradation by chitinases. Furthermore, Knk was also shown to be important for cuticle integrity and laminar organization of the embryonic procuticular chitin in *Drosophila melanogaster* (Moussian et al., 2006) and *T. castaneum* (Chapter 4) (Chaudhari et al., 2011).

In the current study, we describe the identification of two *Knk*-like genes, *TcKnk2* and *TcKnk3*, in the genome of *T. castaneum* and determine their patterns of expression during development and in different tissues. RNA interference (RNAi) studies reveal essential roles of the *Knk*-like genes in embryonic as well as post-embryonic stages of development. The presence of orthologs of *TcKnk2* and *TcKnk3* in several other insect species suggests a conserved function for these proteins during insect cuticle morphogenesis.

## Material and methods

### Insect cultures

*T. castaneum* GA-1 strain was used for all experiments. Insects were reared at 30°C in wheat flour containing 5% brewer's yeast under standard conditions as described previously (Beeman and Stuart, 1990).

### Identification of *TcKnk*-like genes in the *T. castaneum* genome database

A genome-wide TBLASTN search using the amino acid sequence of *T. castaneum* Knk (TcKnk) as a query was carried out at NCBI (<http://www.ncbi.nlm.nih.gov/>) and BeetleBase (<http://beetlebase.org/>). This resulted in identification of two genes, which we have named *T. castaneum Knk-2* (*TcKnk2*) and *T. castaneum Knk-3* (*TcKnk3*).

### Identification of *TcKnk*-like genes in insect genomes

Using the amino acid sequences of TcKnk2 and TcKnk3 as queries, orthologs for TcKnk-like genes were detected in all of the fully sequenced insect genomes. TcKnk2 and TcKnk3 orthologs were detected in *D. melanogaster* (fruit fly); *Anopheles gambiae* (African malaria mosquito); *Aedes aegypti* (yellow fever mosquito); *Culex quinquefasciatus* (southern house mosquito); *Pediculus humanus corporis* (body louse); *T. castaneum* (red flour beetle); *Apis mellifera* (honey bee); *Nasonia vitripennis* (jewel wasp) and *Acyrtosiphon pisum* (pea aphid). No orthologs for TcKnk2 and TcKnk3 were detected in several non-insect arthropod and other invertebrate species including *Daphnia pulex* (water flea), *Ixodes scapularis* (deer tick), *Strongylocentrotus purpuratus* (sea urchin) and *Caenorhabditis elegans* (roundworm).

### Cloning and sequencing of cDNAs for *TcKnk*-like genes

The complete coding sequences of *TcKnk2* and *TcKnk3* were amplified using gene-specific primers (Table 5.1) and cDNA prepared from RNA extracted from different stages of beetle development. 5'- and 3'-RACE reactions were performed to detect the upstream and downstream untranslated regions for both of these genes. Using a pair of primers derived from the predicted full length mRNA, a 1,899 bp *TcKnk2* cDNA was amplified and cloned into pGEMT vector (Promega). Cloning of cDNAs for *TcKnk3* using the same techniques resulted in isolation of two

**Table 5.1 Primer sequences**

<b>Primer</b>	<b>Forward Proimer (5'-3')</b>	<b>Reverse Primer (5'-3')</b>
TcKnk2	CGTGAAGATCAATACGTCGC	GGAGCGTCTCGATAATTGCA
TcKnk2 dsRNA1	TAATACGACTCACTATAGGGTGGCAGCGAAAATCCG	TAATACGACTCACTATAGGGTTGTCCCAGCAACGC
TcKnk2 dsRNA 2	TAATACGACTCACTATAGGGTCATCACCACCATCAC	TAATACGACTCACTATAGGGTGAGTTGGACGACGAG
TcKnk2 R1-5'-In-1	----	CTTCAAAGCCGTCAAGTCTT
TcKnk2 R2-5'-In-2	----	CGAGGACATCGAGATCCAGC
TcKnk2 R3-5'-Out	----	CCTAAGACTGCCGATGGGTA
TcKnk2-F1- 3'-In	----	CGCTCATTCATAAGAGACA
TcKnk2-F1- 3'-Out	----	GCACCTTCACGGCGAAAATC
TcKnk3-FL	ATGGGCCCCATCGTTGCATT	GTTTTGTGTCGTTATTTTTGTG
TcKnk3-5'	ATGGGCCCCATCGTTGCATT	GGCGAAGTGGTTCAGGTGTT
TcKnk3 R1-5'- In-1	----	CGCCGGAGACAGTATTGTTT
TcKnk3 R2-5'-In-2	----	GCGAAATTCTGGGTGGGTCG
TcKnk3 R3-5'-Out	----	CCAGAAGATCGAGCCTTTGA
TcKnk3-F1- 3'-In- 1	GAAGAAAAATGTGAAGATTTTC	----
TcKnk3-F1- 3'-In- 2	GACGGAAGTGTTAAACCTACAG	----
TcKnk3-F1- 3'-Out	CAAGGCGATTGGAAGCCGATG	----
TcKnk3- Exon 1	TAATACGACTCACTATAGGGTGAAAGACATCGAAGG	TAATACGACTCACTATAGGGTTATCGCCGTTTCAGG
TcKnk3- Exon 2 and 3	TAATACGACTCACTATAGGGTCCGGCGCTCCCTATT	TAATACGACTCACTATAGGGTCGTGTGGTGCAGGA
TcKnk3- Exon 5	TAATACGACTCACTATAGGGTCTGACAGTGTTTGA	TAATACGACTCACTATAGGGTCACAGTCGAAGCTGA
TcKnk3- Exon 6	TAATACGACTCACTATAGGGTTCGAAGCTGAACTGC	TAATACGACTCACTATAGGGTCAACTAGTTGCAAAATTAG
TcKnk3- Exon 7 N-ter	TAATACGACTCACTATAGGGTGGCATCTCGGGCGAT	TAATACGACTCACTATAGGGTGACCCGGCAGATCCAC
TcKnk3- Exon 7C-ter	TAATACGACTCACTATAGGGTCCCTTCTACATCACC	TAATACGACTCACTATAGGGTCGGATAAAGACTCC
TcKnk3- Exon 8	TAATACGACTCACTATAGGGTGTTCACGCATCGTT	TAATACGACTCACTATAGGGTCGGACCGAATCAAGG
TcKnk3- Intron 8	TAATACGACTCACTATAGGGTCAAGGGTAAAACCAA	TAATACGACTCACTATAGGGTTACAACAGTTTCAGAAA
TcKnk3- Exon 9	TAATACGACTCACTATAGGGTCCACAGCACTTCGAA	TAATACGACTCACTATAGGGTGAAGCGAGTTTCCG



alternatively spliced variants, namely *TcKnk3-full length (TcKnk3-FL)* and *TcKnk3-5'* of 3,618 bp and 1,212 bp, respectively. A PCR for *TcKnk3* using a forward primer in exon 6 and reverse primer in exon 9 resulted in amplification of a short 3' transcript, for which 5' - UTR regions are still unknown. Sequencing of these cDNA clones was carried out at the DNA sequencing facility at Kansas State University.

### **Phylogenetic analysis of TcKnk-like proteins**

Multiple sequence alignment of TcKnk-like proteins from insects was carried out using the ClustalW software prior to phylogenetic analysis. MEGA 4.0 (Tamura et al., 2007) was used to construct the consensus phylogenetic tree, using the neighbor joining method. To evaluate the branch strength of the phylogenetic tree, bootstrap analysis of 5,000 replications was performed. Bootstrap values are indicated at each branch point on the cladogram.

### **Developmental and tissue-specific expression profile for *TcKnk*-like genes**

To determine the developmental stage-specific expression profile, total RNA was extracted from embryos, young larvae, last instar larvae, pharate pupae, pupae, young adults (0 d-old) and mature adults (10 d-old) using the RNeasy Mini kit (Qiagen). For determination of tissue specificity of expression, total RNA was also isolated from midgut, hindgut and carcass (whole body minus gut) of last instar feeding stage larvae (n=10) according to the manufacturer's instructions. The Superscript III first-strand synthesis system for RT-PCR (Invitrogen) was used to synthesize first-strand cDNA according to the manufacturer's instructions. Gene-specific primers were used to detect each transcript from the prepared sets of cDNAs (Table 5.1). *TcRpS6* (*T. castaneum ribosomal protein S6*) was amplified to serve as an internal loading control for RT-PCR (Arakane et al., 2005a).

### **RNA interference studies**

The two most divergent regions from two different parts of *TcKnk2* gene were selected as targets for RNAi (Table 5.1). A total of nine dsRNAs for three different transcripts of *TcKnk3* were designed by targeting different exons/introns (exon 1, 2, 3, 5, 6, 7-5'-terminal, 7-3'- terminal, 8, 9 and intron 8). Pairs of forward and reverse primers corresponding to these regions with additional T7 promoter sequences at the 5'-ends were synthesized (Table 5.1) and used for the preparation of dsRNAs using an Ampliscribe T7-Flash Transcription Kit (Epicentre

Technologies) as described previously (Arakane et al., 2005b). A control dsRNA for the gene responsible for eye pigmentation named *T. castaneum Vermilion* (*TcVer*) was used as a control. The purified dsRNAs were injected into penultimate instar larvae, last instar larvae and pharate pupae (200 ng per insect, n = 40). After 4-5 d, total RNA was extracted from pools of five insects at either pupa d 3 (for *TcKnk2*) or the pharate adult stage (for *TcKnk3*) for measuring transcript levels by RT-PCR using gene-specific primer-pairs. The remaining insects were observed daily for any visible abnormalities and mortality.

### **Parental RNAi**

For parental RNAi *TcKnk2* or *TcKnk3* (exon 9)-specific dsRNAs were injected into two-week-old mature female adult beetles (n = 20). One day after injection, female beetles were mated with an equal number of male adult beetles. Eggs were collected every four days.

### **Northern blot for detection of *TcKnk3* transcripts**

Total RNA was extracted from insects (n = 4) treated with *TcVer*, *TcKnk3-exon9*- or *TcKnk3-exon5*-specific dsRNAs. Ten µg RNA was then loaded on the gel and transferred onto the nitrocellulose membrane. <sup>32</sup>P-labeled *TcKnk3*-5'- and 3'-terminal DNA probes were designed using the primers listed below and were prepared as described by Nemoto et al. (Nemoto et al., 2010). *TcKnk3*-5'-terminal probe of 551 bp was designed by using exon 1-specific forward primer (5'-ATGGGCCCCATCGTTGCATT-3') and exon 5-specific reverse primer (5'-GCGAAATTCTGGGTGGGTCG- 3'). *TcKnk3*-3'-terminal probe of 1,370 bp was designed by using exon 8-specific forward primer (5'-CACGACAAGTGCGACGAGCA- 3') and exon 9-specific reverse primer (5'-GCCGCGGAAGTTATCAAAGC- 3'). mRNA was then hybridized with <sup>32</sup>P-labeled *TcKnk3*-5'- or 3'-terminal probe at 65°C overnight. High stringency hybridization and washing conditions were employed (Nemoto et al., 2010). Transcripts of differing sizes were then detected after autoradiography using an imaging plate.

### **Chitin content analysis**

*TcKnk2* or *TcKnk3* (exon-9) specific dsRNAs were injected into last instar larvae and pharate pupae (n = 20). Four to five days after injections, insects were collected at pharate pupal and pharate adult stages of development. After *TcKnk3* RNAi of female adult beetles, ~200 embryos were collected. Total chitin content analysis of whole insects or embryos (n = 5) collected at the

indicated stages was performed as described previously (Arakane et al., 2008). GraphPad Prism software was used to plot the graphs and for data analysis.

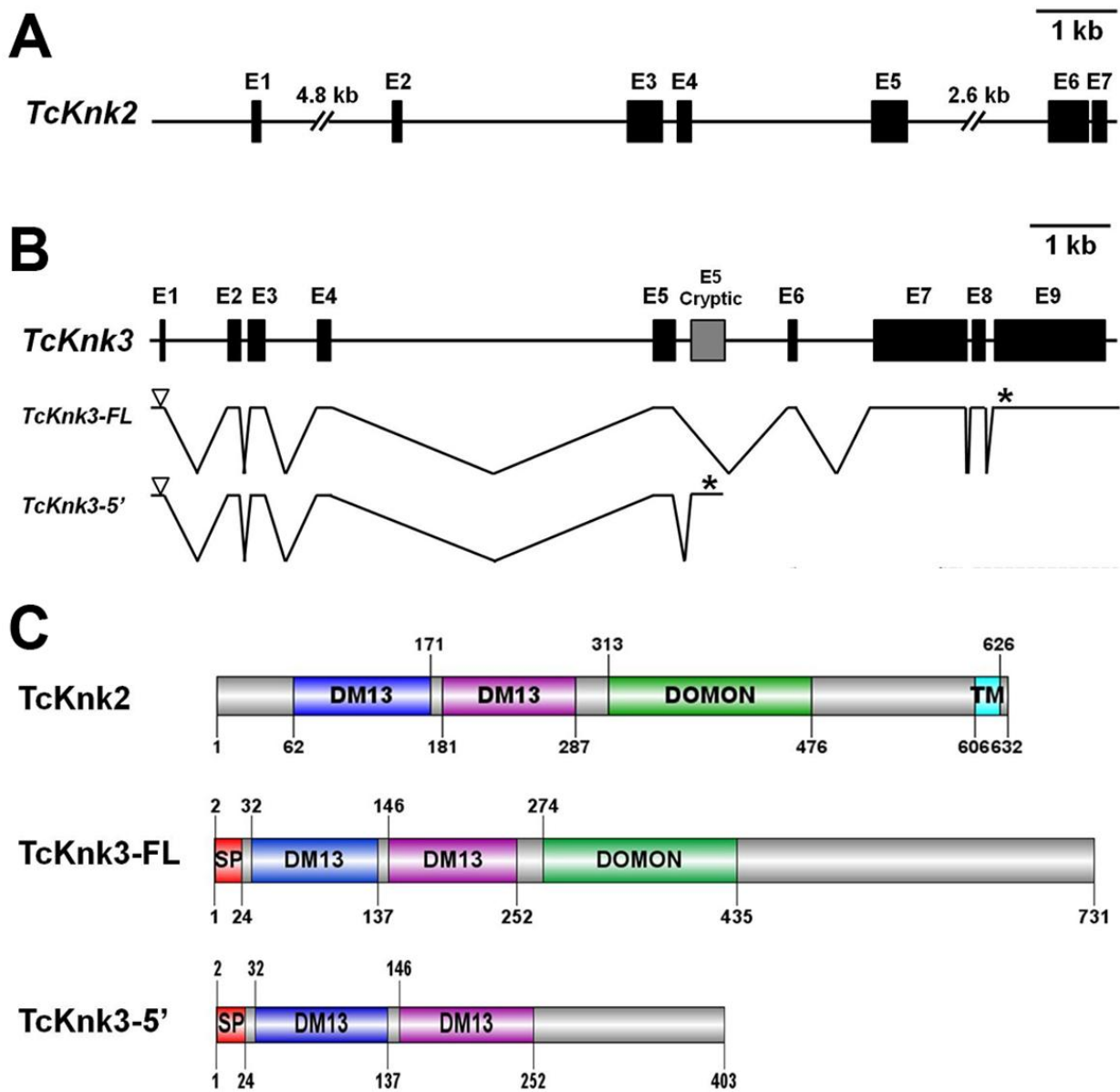
### **Transmission electron microscopic (TEM) analysis**

For TEM analysis insects were injected with *TcKnk2* or *TcKnk3* (exon-9)-specific dsRNAs at pharate pupal stages of development. Five days later, pharate adult insects were collected and fixed overnight at room temperature using a fixative containing 2% para-formaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were then processed as described earlier (Chaudhari et al., 2011) and sectioned to obtain 70 nm thin sections and observed under TEM for final imaging. New adult elytral cuticle, body wall cuticle and trachea isolated at the pharate adult stage was analyzed by TEM.

## **Results**

### **Identification, cloning and sequencing of *TcKnk*-like genes from *T. castaneum* genome**

Putative homologs of *TcKnk* were detected in an extensive genome-wide search by using the NCBI TBLASTN program. The identified *TcKnk*-like genes, which were named *TcKnk2* and *TcKnk3*, both map to linkage group 9, at positions 11.0 cM and 34.1cM, respectively. To verify the gene models for these two predicted *Knk-like* genes, we amplified cDNAs for *TcKnk2* and *TcKnk3* using gene-specific primers (Table 5.1). 5'- and 3'- RACE were performed to determine the upstream and downstream untranslated regions (UTR) for both of these genes. From the 5'- and 3'-RACE sequences, primers were designed to amplify the DNA fragment corresponding to the entire transcribed region for each gene. Comparison of the sequences of these cDNA clones with the genomic sequence allowed us to confirm a gene model for *TcKnk2* that is composed of seven exons capable of encoding a protein of 70.56 kDa with 632 amino acids (Fig 5.1A).



**Figure 5.1 Schematic diagram of the exon–intron organizations and domain architecture of the putative *Tribolium castaneum* Knk-like genes and proteins.**

(A-B) The exon–intron organization of each *TcKnk*-like gene was determined by sequence comparison between genomic sequence and the longest available cDNA sequence. The alternatively spliced forms of *TcKnk3* (*TcKnk3-FL* and *TcKnk3-5'*) are shown in (B). Black closed boxes indicate exons and lines indicate the introns. Gray colored closed box indicates alternatively spliced exon 5 for *TcKnk3-5'*. The open arrowheads represent the start codons and \* indicates the stop codons. (C) Domain architecture of putative TcKnk-like proteins from *Tribolium castaneum*. Domain analysis was done using SMART protein database and graphed using protein domain illustrator program, DOG 1.0.5.

However, 3'-RACE for *TcKnk3* using a forward primer in exon 5 and an oligo-(dT)<sub>18</sub> primer as the reverse primer detected two RT-PCR products that differed substantially in size, suggestive of two alternative polyadenylation sites. Comparison of the *TcKnk3* cDNA longer product sequence with the available gene model indicated presence an additional 55 bp intron between exon 8 and exon 9. The shorter *TcKnk3* 3'-RACE product included a sequence stretch from the middle of intron 5, which contains a stop codon, a polyadenylation signal and a short poly A sequence. This shorter transcript was completely missing all of the downstream exon sequences (Fig 5.1B). From the sequences of these RT-PCR products, we designed a forward primer in exon 1 upstream of the start codon, and reverse primers either in the cryptic exon (exon 5-cryptic) or in exon 9. cDNAs corresponding to the two alternatively spliced/polyadenylated transcripts for *TcKnk3* were cloned and sequenced. Both clones had the first five exons in common and differed only in the 3'-terminal region as a result of the inclusion/exclusion of the cryptic exon within intron 5 of the gene model. Use of this cryptic exon, which we have named E5 cryptic, resulted in the appearance of an in-frame stop codon followed by an alternate polyadenylation site and a shorter transcript with a 3'-poly A tail. Skipping this cryptic exon resulted in a much longer transcript, which included exons 6, 7, 8 and 9, (and, as a consequence, a much longer open-reading frame) and the utilization of a second polyadenylation site downstream of the stop codon in exon 9. These two transcripts derived from the *TcKnk3* gene were named, *TcKnk3*-full length (*TcKnk3-FL*) and *TcKnk3-5'* (Fig 5.1B). Comparison of cDNA clones for *TcKnk3-FL* and *TcKnk3-5'* with the genomic sequence confirmed the presence of a total of 10 exons (including cryptic exon 5) capable of encoding an 81 kDa protein by the *TcKnk3-FL* transcript and a 46 kDa protein by the *TcKnk3-5'* transcript, respectively.

### **Domain organization and phylogenetic analysis of TcKnk2 and TcKnk3 proteins**

Both DmKnk and its orthologous protein, TcKnk, are predicted to encode putative C-terminal GPI-anchored proteins containing three domains, namely two N-terminal tandem DM13 domains and a central dopamine  $\beta$ -monooxygenase N-terminal-like (DOMON) domain (Chaudhari et al., 2011; Moussian et al., 2006). As with multiple extracellular adhesion proteins, both DOMON and DM13 domains are enriched in  $\beta$ -sheet-folds that are predicted to interact with sugar or heme ligands and/or participate in redox reactions that could potentially promote oxidative cross-

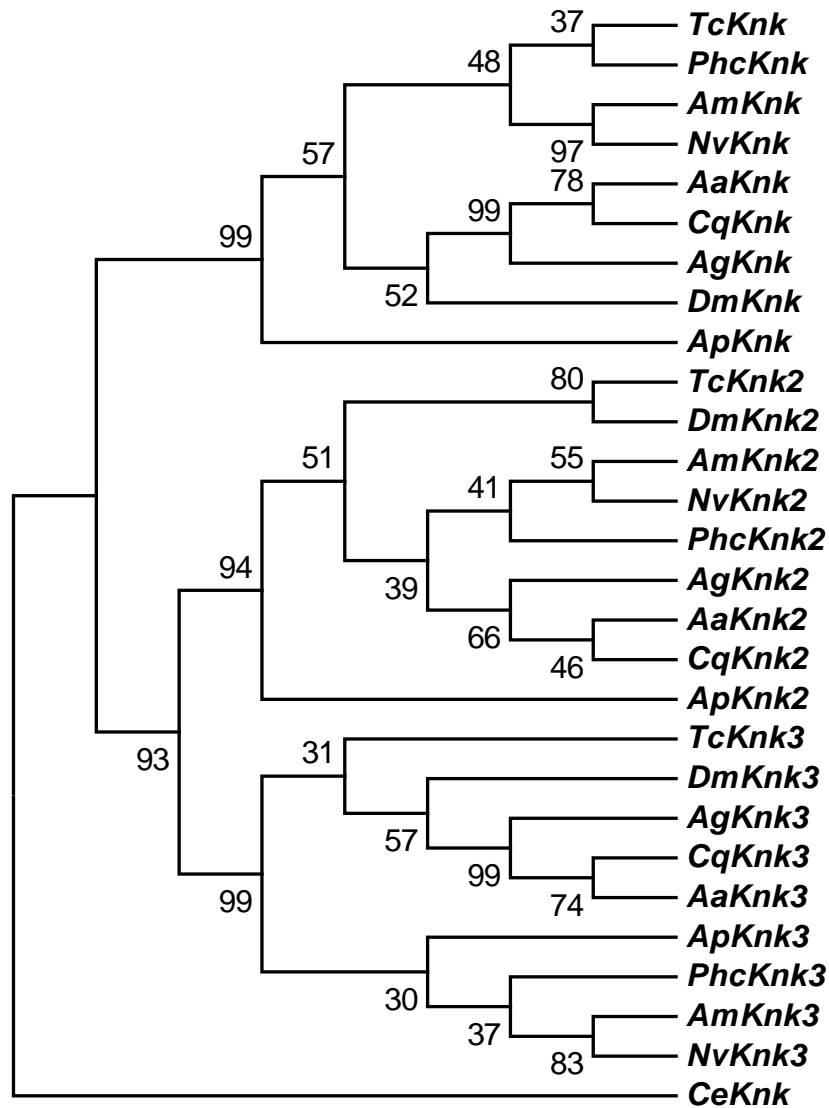
linking of proteins (Aravind, 2001; Iyer et al., 2007). Although it is unclear at present whether TcKnk possesses a redox functions, this protein was shown to have strong affinity for chitin (Chaudhari et al., 2011). The domain organization of TcKnk-like proteins was performed using the SMART protein database and graphed using protein domain illustrator program, DOG 1.0.5. Domain analysis indicated *TcKnk2* encodes a protein with two DM13 domains and a DOMON domain with a predicted C-terminal transmembrane segment (Fig 5.1C). TcKnk3-FL protein is predicted to have an N-terminal signal peptide, two DM13 domains and a DOMON domain, but no predicted GPI anchor or transmembrane segment at the C-terminal end (Fig 5.1C). TcKnk3-5' protein is predicted to have an N-terminal signal peptide and two DM13 domains only (Fig 5.1C). The similarities in the domain architectures of TcKnk-like proteins in comparison with TcKnk indicate similar functions in cuticulogenesis of the beetle (Appendix C).

### **Knk-like proteins are present in several orders of insects**

The identification of two genes encoding TcKnk-like proteins in the *T. castaneum* genome prompted us to investigate whether *Knk-like* genes are present in other insect orders as well. A search of genomes of several insects with fully sequenced genomes including those of *D. melanogaster*, *A. gambiae*, *A. aegypti*, *C. quinquefasciatus*, *A. mellifera*, *A. pisum*, *N. vitripennis* and *P. humanus corporis* indicated that orthologs of TcKnk2 and TcKnk3 are indeed present in these genomes. Conservation of TcKnk-like proteins in different insect species indicates a central role for these proteins in the development of the chitinous exoskeleton in many insect species and possibly other arthropods (Fig 5.2). However, it should be pointed out that our search failed to identify orthologs in other arthropods such as the water flea and the deer tick, nor was it found in the sea urchin and nematode which also synthesize chitin.

### **Developmental stage and tissue-specific expression profiles of *TcKnk2* and *TcKnk3***

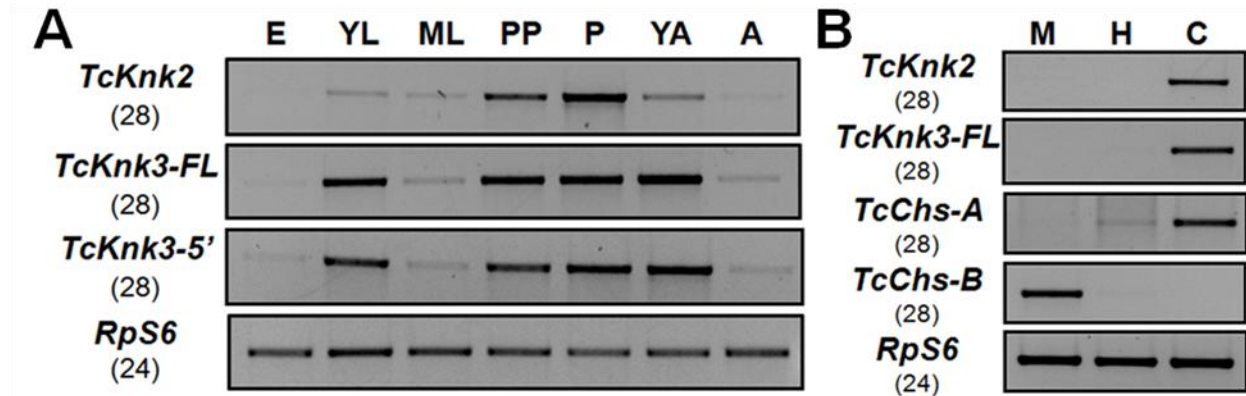
To determine the differences in the expression patterns of *TcKnk*-like genes during *Tribolium* development, we analyzed the levels of *TcKnk2*, *TcKnk3-FL* and *TcKnk3-5'* transcripts using as template cDNAs prepared from RNA extracted from embryos, young larvae, mature larvae, pharate pupae, pupae, young adults (0 d-old) and mature adults (10 d-old). *TcKnk2* transcripts



**Figure 5.2 Phylogenetic analysis of insect TcKnk2 and TcKnk3.**

MEGA 4.0 was used to construct the phylogenetic tree using the neighbor joining method (Tamura et al., 2007). Bootstrap analyses from 5000 replications are shown by each branch. *Drosophila melanogaster* (fruit fly) (Knk2, NP\_001097889.2; Knk3, NP\_001027171.1); *Anopheles gambiae* (African malaria mosquito) (Knk2, XP\_308115.4; Knk3, XP\_313250.2); *Aedes aegypti* (yellow fever mosquito) (Knk2, XP\_001657415.1; Knk3, XP\_001657668.1); *Culex quinquefasciatus* (southern house mosquito) (Knk2, XP\_001845444.1; Knk3, XP\_001864909.1); *Pediculus humanus corporis* (head louse) (Knk2, XP\_002429468.1; Knk3, XP\_002425509.1); *Tribolium castaneum* (red flour beetle) (Knk2, XP\_973211.1; Knk3, XP\_968712.2); *Apis mellifera* (honey bee) (Knk2, XP\_393508.4; Knk3, XP\_003250319.1); *Nasonia vitripennis* (parasitic wasp) (Knk2, XP\_001602771.1; Knk3, XP\_001606495.2) and *Acyrtosiphon pisum* (pea aphid) (Knk2, XP\_001946688.2; Knk3, XP\_003241759.1).

were detected in all stages of insect development with highest expression levels in the pupal stage (Fig 5.3A), while *TcKnk3-FL* as well as *TcKnk3-5'* transcripts were abundant in young larval, pharate pupal, pupal and young adult stages of development (Fig 5.3A). We performed RT-PCR using cDNAs prepared from midgut, hindgut and carcass as templates to further understand the tissue-specific expression patterns of *TcKnk2* and *TcKnk3-FL*. Transcripts for both these genes were detected only in carcass and not in midgut tissues, consistent with a role for these proteins in cuticle-forming tissues but not in PM-forming tissues (Fig 5.3B). *TcChs-A* and *TcChs-B* expression was also measured for monitoring the contamination of cDNA templates with RNA from non-targeted tissues. RT-PCR for *TcRpS6* was used as an internal loading control in both the experiments.



**Figure 5.3 Developmental stage- and tissue-specific expression of *TcKnk*-like genes by RT-PCR.**

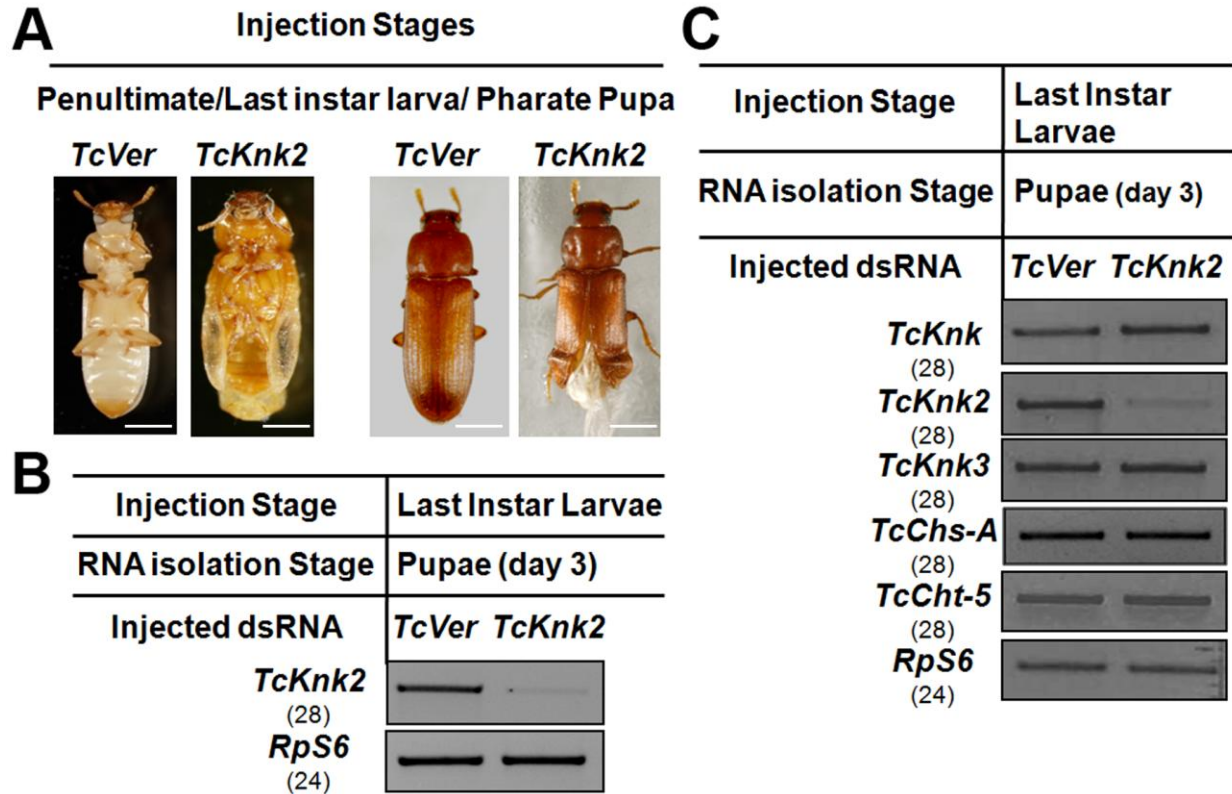
(A) Developmental expression profiles of *TcKnk2*, *TcKnk3-FL* and *TcKnk3-5'* transcripts. cDNAs were prepared from total RNA extracted from whole insects at several developmental stages including E, embryos; YL, young larvae (penultimate instar or younger); LL, last-instar larvae; PP, pharate pupae; P, pupae; YA, young adults (0 d- old); A, mature adults (10 d-old). (B) Tissue-specific expression of *TcKnk*-like genes in the feeding stage last instar larvae. M, midgut; H, hindgut; and C, carcass (whole body without gut). *T. castaneum* ribosomal protein-S6 (*TcRpS6*) was used as internal loading control for RT-PCR. Results are from 28 and 24 cycles of RT-PCR for *TcKnk-like genes* and *TcRpS6*, respectively.



### ***TcKnk2* contributes to pupal-adult molt of *T. castaneum* development**

To determine the role of *TcKnk2* in *T. castaneum* development and molting, we injected young larvae, last instar larvae or pharate pupae with either of two *TcKnk2* dsRNAs (*dsTcKnk2*), targeting two non-overlapping regions of the same gene. *dsTcVer*, a dsRNA targeted specifically against *tryptophan oxygenase*, a gene required for eye pigmentation in *Tribolium*, was used as a control. All *TcKnk2* dsRNA-treated insects from young larval, last instar larval and pharate pupal stages exhibited lethal phenotype only at the pharate adult stage of development with mortality rate of ~55%. From the molted insects ~30% adults showed hypomorphic phenotypes (Fig 5.4A). Insects arrested at the pharate adult stage exhibited a clear molting defect as a result of inability to shed the old pupal cuticle. Adults with the hypomorphic phenotype showed 100% mortality within 10-15 d of adult emergence, whereas other adults were unaffected and had normal lifespan. Adult hypomorphs resulting from RNA interference (RNAi) for *TcKnk2* had warped elytral tips and misfolded wings (Fig 5.4A). We confirmed the specificity and effectiveness of *TcKnk2* dsRNA treatment by RT-PCR using cDNA prepared from RNA isolated from pupal day 3, that is, collected four days after dsRNA treatment. *TcKnk2* transcript levels were significantly down-regulated after *TcKnk2* dsRNA treatment in comparison with control dsRNA *TcVer*-injected insects (Fig 5.4B).

The molting defect observed at the pharate adult stage after *TcKnk2* RNAi was similar to that following *TcKnk* or *TcChs-A* RNAi. To determine the cross-knockdown of *TcKnk*-like transcripts or those involved in chitin metabolism such as *chitin synthase-A* (*TcChs-A*) or *chitinase-5* (*TcCht-5*), we performed RT-PCR using cDNA prepared from RNA extracted from 3-d-old pupae (n = 4) after *TcKnk2* dsRNA treatment. RT-PCR using gene-specific primers confirmed specific knockdown of *TcKnk2* transcripts upon *TcKnk2* RNAi with no effect on the transcript levels for *TcKnk*, *TcKnk3*, *TcChs-A* or *TcCht-5* (Fig 5.4C).



**Figure 5.4 Effect *TcKnk2* dsRNA-treatment on the development of *T. castaneum*.**

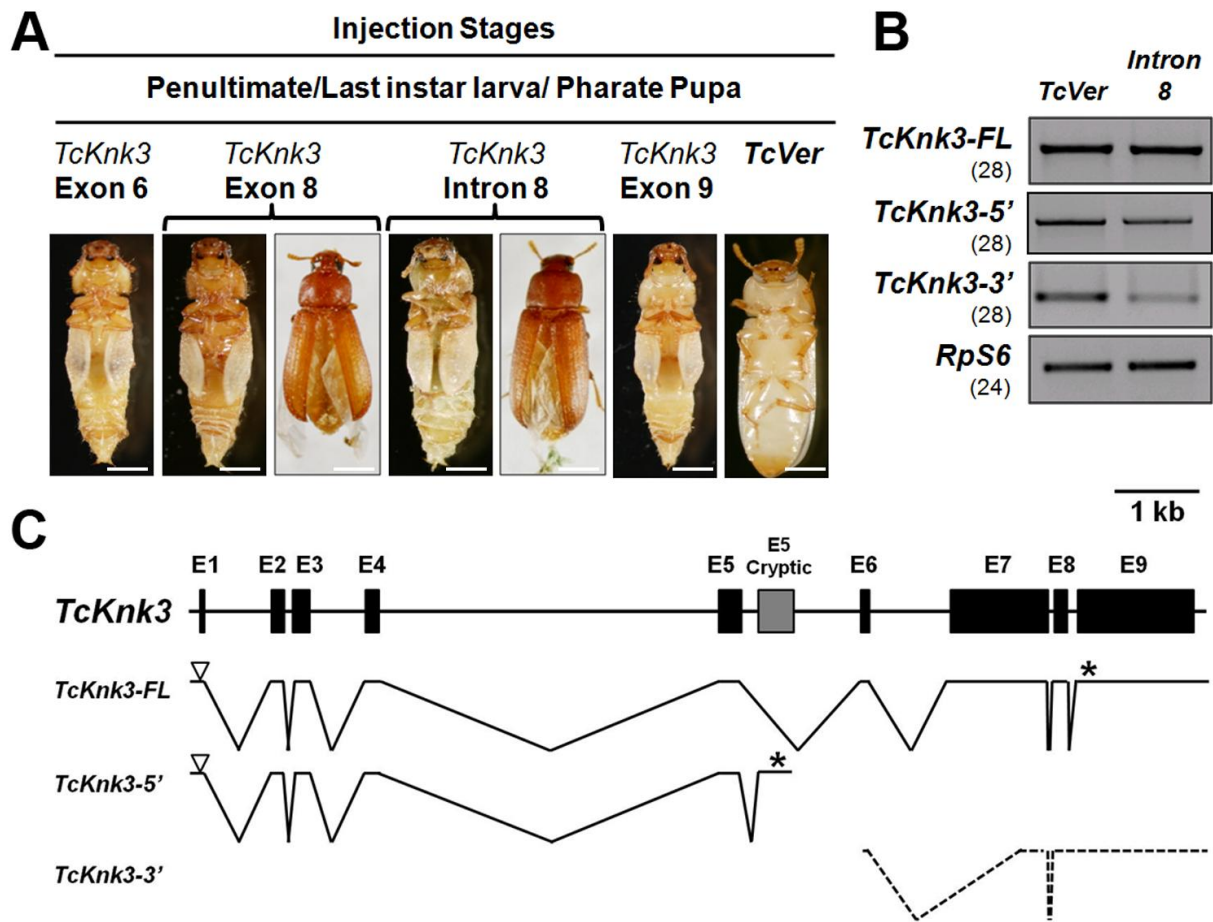
(A) Injection of dsRNA for *TcKnk2* into penultimate instar larvae, last instar larvae and pharate pupae led to lethal phenotype at pupal-adult molt (~55%) and ~15% adult hypomorphic phenotype with split elytra. Scale bar = 1 mm. (B) Specific down-regulation of *TcKnk2* transcripts by RNAi. dsRNAs (200 ng per insect) for *TcKnk2* were injected into pharate pupae. Three days after injection, total RNA was extracted from whole insects of at pupal stage day 3 (n = 3) and used for cDNA synthesis. (C) Specificity of *TcKnk2* dsRNA-treatment. Effect of *TcKnk2* dsRNA-treatment on transcripts for *TcKnk*, *TcKnk3* and other genes involved in chitin metabolism such as *TcChs-A* and *TcCht-5* was checked by RT-PCR. *T. castaneum ribosomal protein-S6* (*TcRPS6*) was used as internal loading control. dsRNA for *T. castaneum Vermilion* (*TcVer*) was injected as a control.

**RNAi reveals that the 3'-half of the *TcKnk3* gene may be transcribed to yield a shorter transcript essential for adult morphogenesis.**

To determine functions of both *TcKnk3-FL* and *TcKnk3-5'* transcripts in the development of *Tribolium*, dsRNAs corresponding to regions in exon 9 or 5 were designed to down-regulate only the longer transcript or both, respectively. These dsRNAs were injected at young larval, last

instar larval or pharate pupal stages of *Tribolium* development. Despite substantial depletion of both *TcKnk3-FL* and *TcKnk3-5'* transcripts by exon 5-specific dsRNA treatment, no visible phenotype was observed, as all insects injected with dsRNA at any stage of development developed into adults without molting defects or visible abnormalities (Fig 5.6). Unexpectedly, injection of exon 9-specific dsRNA that also led to a similar depletion of the *TcKnk3-FL* transcripts resulted in 100% mortality at the pharate adult stage of molting. No molting defects or abnormal phenotypes were observed during the earlier stages of development including larval-larval and larval-pupal molts (Fig 5.5A). To understand the differences in the RNAi results from exon 5 or 9 dsRNA treatment, we injected insects at different stages of development with dsRNAs specific for different exons including exons 1 (with the 5'-UTR region), 2 and 3 (spanning both exons), 6, 7-5'-terminal half, 7-3-terminal half or exon 8. Exon 1, 2, 3, 7-5'-terminal or 7-3'-terminal-specific dsRNA-treated insects developed normally into adults in comparison with control *dsRNA TcVer*-treated insects. However, both exon 6 and exon 8 dsRNA-treated insects showed molting defects similar to those seen with exon 9 RNAi, resulting in 100% and 82% mortality, respectively, at the pharate adult stage of development (Fig 5.5A). Even the survivors of the exon 8-specific dsRNA treatment (18%) exhibited a hypomorphic phenotype with split elytra as adults (Fig 5.5A).

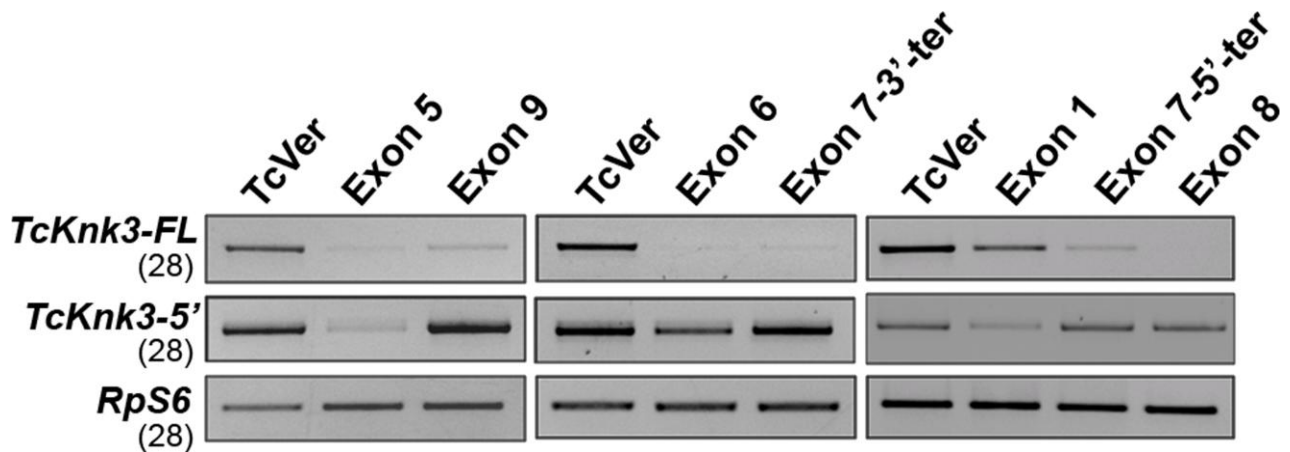
The finding that RNAi for the *TcKnk3-5'* transcript using different dsRNAs showed no visible phenotype or molting defect even though the corresponding mRNA levels were severely depleted indicated that this short transcript is non-essential for insect development and/or molting. However, the consequences of depletion of the *TcKnk3* full-length transcript depended on the targeted exon. Significant knockdown of *TcKnk3-FL* transcript using dsRNAs specific for exons 1, 2, 3 and 5 did not produce any phenotype (Fig 5.6, Table 5.1), whereas knockdown of the same transcript using dsRNAs specific for exons 6, 8 and 9 showed molting arrest at the pupal-adult molt (Fig 5.5A), indicating the possibility of the presence of another transcript derived exclusively from the 3' region of the *TcKnk3* gene that lacked sequences corresponding to several of the 5'-terminal exons. We hypothesized that this putative 3' transcript must include at least parts of exons 6, 8 and 9 but not exon 7, and named it *TcKnk3-3'*. In order to clone this *TcKnk3-3'* transcript, we performed a 5'-RACE and a 3'-RACE using exon 8-specific forward primers and exon 8-specific reverse primers (Table 5.2).



**Figure 5.5 Effect of *TcKnk3* dsRNA-treatment on the development of *T. castaneum*.**

(A) dsRNAs specific for different exons and intron of *TcKnk3* (exon 6, exon 8, exon 9 and intron 8) were injected into penultimate instar larvae, late instar larvae and pharate pupae. All injected insects showed ~60-100% lethal phenotype at pupal-adult molt. Exon 8 and intron 8 dsRNA-treatment led to adult hypomorphic phenotype with split elytra. Scale bar = 1 mm. (B) Specific down-regulation of *TcKnk3-3'* transcripts by RNAi. dsRNA (200 ng per insect) for *TcKnk3-3'* (corresponding to intron 8) was injected into pharate pupae. Five days after injection, total RNA was extracted from pharate adult insects (n = 3) and used for cDNA synthesis. Depletion of different transcripts was checked by using transcript-specific primer pairs (Table 2). *TcRPS6* was used as internal loading control. *TcVer* dsRNA was injected as a control. (C) Schematic representation of different transcripts from *TcKnk3* gene. *TcKnk3-3'* outline was inferred from the sequences of a partial clone and 3'-and 5'-RACE products, and is shown by dotted lines.

Although the start site for this transcript is still undetermined, 3'-RACE detected a poly-A signal at the end of exon 9 as predicted by the gene model. RT-PCR using a forward primer in exon 6 and reverse primer in exon 9 resulted in cDNA cloning of a partial *TcKnk3-3'* transcript which included most of exon 6, a part of exon 7 (324 nucleotides corresponding to the 3'-end of exon 7 and no intron 7 sequences), all of exon 8, all of intron 8 and all of exon 9 (Fig 5.5C). As this partial *TcKnk3-3'* cDNA had the entire 55 bp-long sequence of intron 8, we tested for the possible presence of an mRNA with this intron sequence incorporated in it utilizing RNAi experiments. We made a dsRNA corresponding to this intron region (and no flanking exon 8 and exon 9 sequences) and injected this intron 8-specific dsRNA into insects at different stages of development. About 66% of the injected insects exhibited pharate adult stage mortality and 15% of the surviving adults had a hypomorphic phenotype similar to that observed after exon 6, 8 and 9 dsRNA treatment (Fig 5.5A).



**Figure 5.6 Specificity of dsRNA-mediated down-regulation of *TcKnk3* transcripts.**

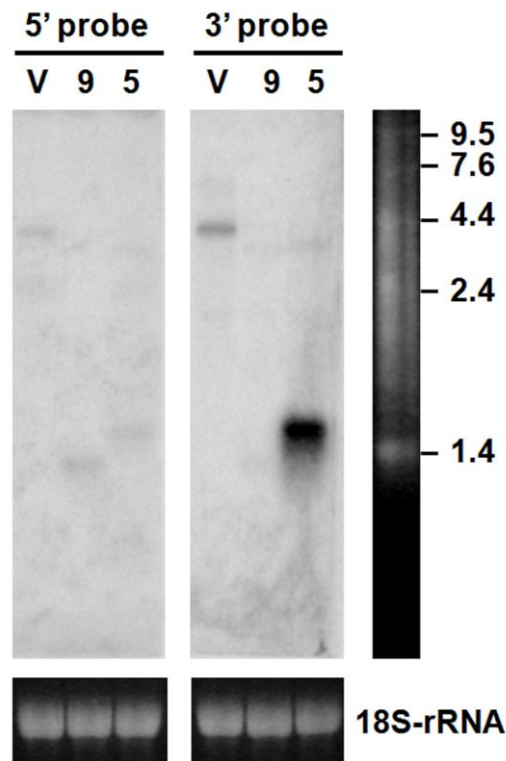
Levels of *TcKnk3-FL* and *TcKnk3-5'* transcripts after treatments with different dsRNA were determined by RT-PCR (28 cycles) using appropriate primers designed to amplify the full length or the 5'-Knk cDNA products. Indicated dsRNAs were injected into pharate pupal insects. Five days post-injection RNA was collected from pharate adult insects for cDNA preparations. *TcRpS6* (24 cycles) was used as an internal loading control for RT-PCR. TcVer dsRNA treatment was used as a control.

To determine the specificity and effectiveness of RNAi, we examined the levels of *TcKnk3-FL*, *TcKnk3-5'* and *TcKnk3-3'* transcripts after each dsRNA treatment using cDNAs prepared from RNA extracted from pharate adult insects four days after injections. Significant depletion of each transcript was observed with each dsRNA treatment (Fig 5.6). The levels of *TcKnk3-FL* transcripts were significantly reduced after treatment with each of the dsRNAs. More importantly, *TcKnk3-3'* transcripts were depleted in intron 8-specific dsRNA-treated insects, indicating the inclusion of this intron in the CDS of a still-uncharacterized transcript that is expressed during development of *Tribolium* (Fig 5.5B). The dsRNA for intron 8 did not appear to reduce the levels of the full-length transcripts, indicating that this dsRNA specifically depletes the *TcKnk3-3'* transcripts, but not the other two transcripts from the same gene (Fig 5.5B). Taken together with the mortality and phenotypic effects of RNAi and transcript depletion of each of the three transcripts of *TcKnk*, these experiments suggest that the *TcKnk3-3'* transcripts are essential for pupal-to-adult development of *T. castaneum*.

### **Detection of *TcKnk3-3'* transcripts by northern blot analysis**

To determine the start and stop sites of the *TcKnk3-3'* transcript, we performed 5'- and 3'-RACE using as template RNA isolated from pharate adults that had been previously injected with dsRNA for exon 5. This treatment was expected to deplete the full length and the 5'-transcripts of *TcKnk3* and, as a result, enrichment of *TcKnk3-3'* transcripts in total RNA preparations. The 3'-RACE using a forward primer in exon 8 yielded a cDNA product that was 782 nucleotides long and included the predicted polyadenylation signal present in the 1,379 nt-long exon 9 of the gene model. The size and sequence of the 3'-RACE product indicated that this transcript has a translational termination site different from the full-length transcript predicted by the gene model (Fig 5.5C). We were unable to clone the cDNA fragment corresponding to the complete *TcKnk3-3'* transcript, presumably due to the low concentration of these transcripts in this template and lack of information about where the promoter for this transcript lies. To directly confirm the presence of this transcript, we performed a northern blot analysis using <sup>32</sup>P-labeled probes derived from the 5'- and 3'-halves of the *TcKnk3* gene, designed to specifically detect only the 5'-transcript or the 3'-transcript, respectively. Either probe should detect the full-length transcript. The 5'- probe was synthesized by amplifying a region of 551 bp using a forward

primer in exon 1 and reverse primer in exon 5 (Table 5.2). The 3'-probe was synthesized by amplifying a 1,370 bp region using a forward primer in exon 8 and a reverse primer in exon 9. Aliquots of 10 µg of total RNA extracted from insects injected with control (*TcVer*), *TcKnk3* exon 9 or *TcKnk3* exon 5 dsRNA were used for northern blot analysis. Hybridization of the membrane with the <sup>32</sup>P-labeled 5'-probe detected a ~3.6 kb transcript in control RNA (*TcVer* dsRNA-treated) corresponding to the size of *TcKnk3-FL* transcript (Fig 5.7).



**Figure 5.7 Northern blot analysis of *TcKnk3* transcripts using <sup>32</sup>P-labeled 5'-terminal and 3'-terminal probes.**

Ten µg of extracted RNA from V, *TcVer*; 9, *TcKnk3*-exon 9; 5, *TcKnk3*-exon 5 specific dsRNA-treated pharate adult insects was loaded onto 1.5% agarose gel, transferred on to the nitrocellulose membrane and hybridized with <sup>32</sup>P labeled 5'-terminal or 3'-terminal probe as described in Materials and Methods.

In RNA from exon 9 dsRNA-treated animals, 5'-probe detected only the shorter *TcKnk3-5'* transcript of ~1.2 kb and not the *TcKnk3-FL* transcript, as expected. In RNA from animals treated with dsRNA for exon 5, neither the full length 3.6 kb transcript nor the *TcKnk3-5'*

transcript (1.2 kb) were detectable, again as expected. Surprisingly, a ~ 1.5 kb transcript was detected, which is the expected size for the *TcKnk3-3'* transcript (Fig 5.8). The identity of this band was confirmed by the results of hybridization with the 3'-specific probe. The same band hybridizes much more strongly with the 3'-probe (right panel), suggesting that the faint band seen in autoradiography with the 5'- probe is due to a low level of non-specific (or cross) hybridization. The 3'-probe also detects the full-length transcript in the control RNA and its level is depleted after administration of exon 9-specific dsRNA as expected. Compared to the control RNA in which the 3'-transcripts are undetectable, there was substantial up-regulation of the steady-state levels of this 1.5 kb transcript when the transcripts for the full length and 5'-*TcKnk3* transcripts were down-regulated by exon 5-specific dsRNA (Fig 5.7). These results confirm that the *TcKnk3* gene has the potential to yield three alternatively spliced transcripts under different conditions.

### >*TcKnk3-3'* transcript

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CGAAGCTGAAGTGGCGAAGTTTGTACGATGACCTGGCATTGAGGTGCGATGGGCGGTTTCGACCCTGAGGTCGCGGCCAAATACC
ATCCCTTCTACATCACCGATGATCCTGTGGGAGGATACGAGTACAAAACCCCGAAGAGAAGAAAAATGTGAAGATTTTCGCCGGC
GCAATTGCCGGGAAGGACGGAAGTGTAAACCTACAGGAACGGGAAGACTGTGCCACTGGACCCAGCAAGGCGATTTGGAAGCCGA
TGATTTTGTAGTCGTTTGGGGCTTATCAAAGAACACTCGAGTTAAAATGTGACCAAGGCGAGCCTGGAGTCATCCAATGGACTCCGG
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ACCCCATATCAACAAACTTTCAAACCTAGCCACTTTTAGAACCTATTGGGGAGGATTTTCAGGCTTAAATCTATACCTTCAGAAGC
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TAGAGACCGCCGAGCGGTTCAACAAAACCTGAGTCCGATAAGACATCAAAGAGGGGTGTAGGCCCGAGTTCGTGGTTATCTAATG
CTTTTTTATTTTAAACGATTGTTAAATAGTTTAGTAAGTTTGTGTGCTTATTTGTGTAATAAAACAATTTGTAGTTGTAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAGTACTAGTCGACGCGTGGCCA

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### Figure 5.8 Sequence for *TcKnk3-3'* transcript.

RT-PCR for *TcKnk3-3'* transcript using exon 6 specific forward primer and a exon 9-specific reverse primer yielded a short transcript that covered part of exon 6 (blue), part of exon 7 (red), complete exon 8 (green), complete intron 8 (black) and part of exon 9 (pink). The sequences corresponding to (or complementary to) the primers used for amplification are underlined.

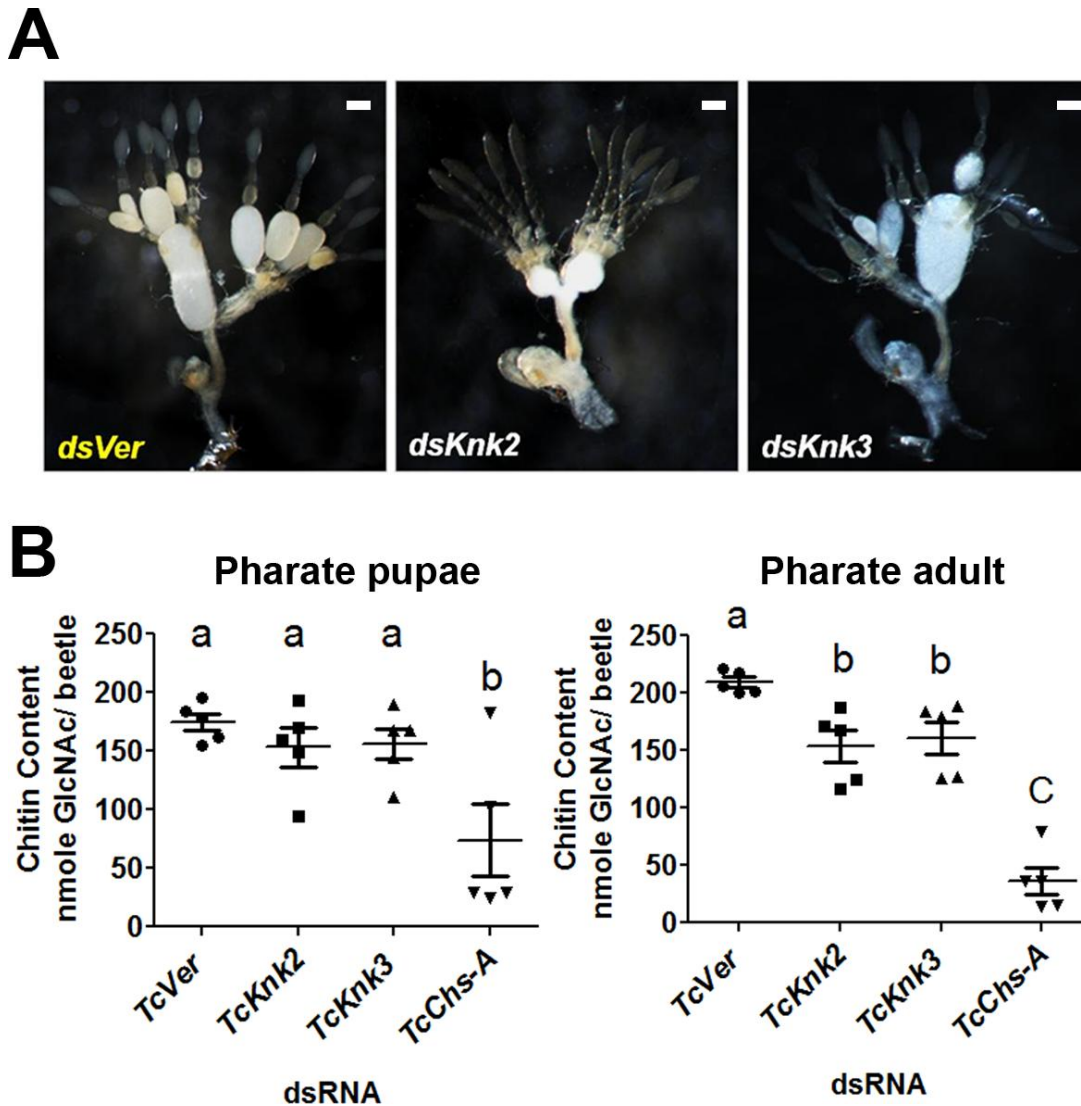


### **Role of *TcKnk*-like genes in embryonic development of *T. castaneum***

TcKnk has been shown to be a chitin-binding protein that binds to chitin in the newly forming procuticle and to protect it from degradation by chitinases. It also helps to organize the chitin into a laminar matrix (Chaudhari et al., 2011). In addition to its essential role during every molt, *TcKnk* is also required for serosal cuticle organization during embryonic development, and is therefore essential for egg hatching in *Tribolium*, without affecting oocyte maturation or the fecundity of the adult females (see Chapter 4). In order to understand the importance of *TcKnk*-like genes in development of *T. castaneum* embryos, we injected 2-week-old female beetles with *TcKnk2 dsRNA* or *TcKnk3* (exon 9-specific) dsRNAs. One day after dsRNA treatment, female beetles were mated with an equal number of male beetles followed by egg collection to monitor the rate of survival and percent egg hatching in comparison with *TcVer* dsRNA-treated control insects. *TcKnk2* dsRNA-treated female adult beetles did not lay any eggs, while *TcKnk3* (exon 9-specific) dsRNA treatment showed no effect on number of eggs laid and hatching percentage in comparison with control females. To determine the cause of the oviposition defect in females following *TcKnk2* RNAi, we dissected out ovaries from *TcKnk2* dsRNA-treated adult females ten days after injection. Along with depletion of *TcKnk2* transcripts, we observed defective oocyte development and necrotic changes in the ovarioles (Fig 5.9A). *TcKnk3*-dsRNA-treated ovaries developed normally in comparison with control *TcVer* (Fig 5.9A). Results from these parental RNAi experiments indicate an important role for *TcKnk2* in oocyte development in *T. castaneum*.

### **TcKnk2 and TcKnk3 are required for chitin maintenance and integrity of procuticular chitin in *T. castaneum***

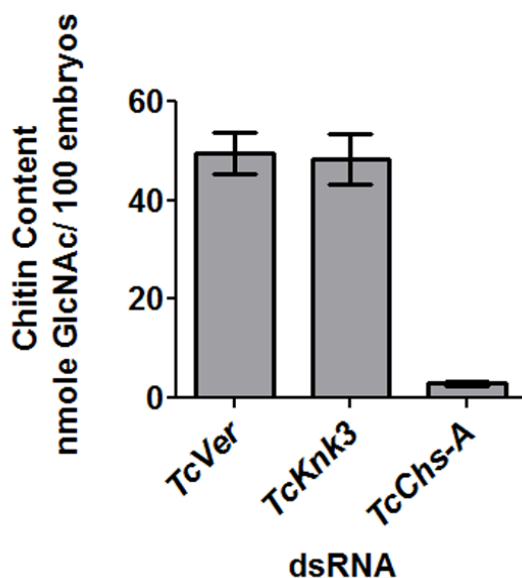
Our recent work has uncovered an important role for TcKnk in protection of procuticular chitin from chitinases. TcKnk has been shown to be important both for the maintenance of chitin levels and its laminar organization in the procuticle (Chaudhari et al., 2011; Moussian et al., 2006). To determine whether *TcKnk*-like genes have any roles in cuticular chitin maintenance, we performed total chitin content analysis of larvae treated with *TcKnk2*- and *TcKnk3* (exon-9)-specific dsRNAs.



**Figure 5.9 Effect of *TcKnk2* and *TcKnk3* dsRNA treatment on ovaries and cuticular chitin content of *T. castaneum*.**

(A) *TcKnk2* (*dsKnk2*) and *TcKnk3* (exon 9) (*dsKnk3*)-specific dsRNAs were injected into two week-old mature females and ovaries were obtained by dissection after ten days. Oocyte development and ovarian structures were compared with those from control *TcVer* dsRNA-treated females (*dsVer*). Scale bar = 1 mm. (B) *TcKnk2* or *TcKnk3* (exon-9)-specific dsRNA was injected into late instar larvae and pharate pupae. Four to five days after injections, pharate pupal and pharate adult (n = 5) insects were collected for chitin content analysis by a modified Morgan–Elson and reported as mean ± SE. dsRNA for *TcVer* and *TcChs-A* were injected as negative and positive controls, respectively. Statistical significance was computed by the Student’s *t*-test. Means identified by different letters are significantly different at  $p < 0.05$ .

Insects were collected at pharate pupal and pharate adult stages of development four to five days after dsRNA injections into either last instar larvae or pharate pupae. There was a significant decrease in chitin content after either *TcKnk2* or *TcKnk3* dsRNA treatment at pharate adult stage of development, indicating an essential role for these two genes in post-embryonic cuticular chitin level maintenance specifically at the pharate adult stage of development (Fig 5.9B). Furthermore, total chitin content analysis of embryos laid by female beetles after *TcKnk3* RNAi showed no significant changes in chitin level when compared with embryos from control insects (*TcVer*), indicating no important role for this gene in embryonic cuticular chitin maintenance (Fig 5.10).

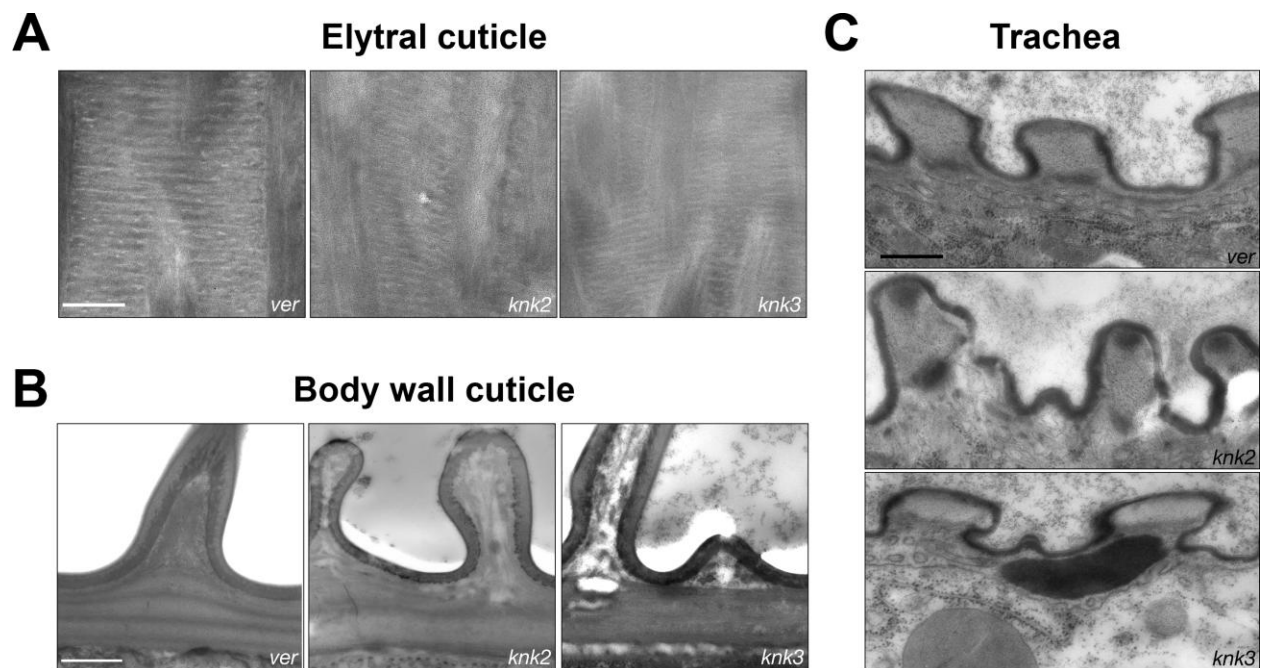


**Figure 5.10 Effect of *TcKnk3* dsRNA-treatment on embryonic chitin content.**

*TcKnk3*-specific dsRNA was injected into two week-old female adult beetles. Approximately 200 embryos were collected for chitin content analysis by a modified Morgan–Elson method and reported as mean  $\pm$  SE ( $n = 5$  each). Embryos collected from *TcVer* and *TcChs-A*-dsRNA treated insects were used as controls. Statistical significance was computed by the Student's *t*-test. Means identified by different letters are significantly different at  $p < 0.05$ .

To further determine the role(s) of *TcKnk*-like genes in organization of the procuticular chitin, we performed transmission electron microscopic (TEM) analysis of pharate adult elytral cuticle, body wall cuticle and the trachea. TEM of elytra from control (*TcVer*)-dsRNA treated

insects showed a horizontally arranged laminar organization of the procuticular chitin, that is, parallel to the apical surface of the epidermal cell. However, no significant difference in the laminar architecture of elytral procuticular chitin in comparison with control insects was seen after *TcKnk2* and *TcKnk3* dsRNA-treatment (Fig 5.11). However, TEM analysis of body wall denticle cuticle revealed an amorphous procuticle in the absence of TcKnk2 and TcKnk3. Similarly, TEM analysis of tracheae showed accumulation of electron dense material at the tip of the taenidia upon *TcKnk2* and *TcKnk3* dsRNA treatment. It is interesting to observe that *TcKnk2* and *TcKnk3* defects are associated with structures where chitin is not organized in a laminar fashion. The lethal phenotypes observed at pharate adult stage of molting after *TcKnk2* and *TcKnk3* RNAi could result from loss of integrity in body wall and failure to develop tracheae normally. Chitin content and TEM analysis revealed significant roles for TcKnk-like proteins in chitin maintenance and cuticle integrity in the body wall denticles and tracheae.



**Figure 5.11 Effect of *TcKnk2* and *TcKnk3* dsRNA treatment on procuticle structure of *T. castaneum*.**

Transmission electron microscopic analysis of *TcKnk2* and *TcKnk3* (exon 9)-specific dsRNA-treated pharate adult elytra, body wall and trachea. dsRNA for *TcVer* was injected as a control. Scale bar for all sections in A, B and C = 500 nm.

## Discussion

Our recent study has demonstrated an important role for TcKnk in protecting the newly synthesized procuticular chitin from degradation by active chitinases by colocalizing with chitin in the procuticle (Chaudhari et al., 2011). Additionally, TcKnk was shown to bind to chitin and to be important for the laminar organization of procuticular chitin in embryonic and post-embryonic stages of *T. castaneum* development. Protein domain analysis of TcKnk has shown that this protein has a C-terminal GPI-anchor along with two N-terminal DM13 domains and a central DOMON domain. Although the functions of the DM13 and DOMON domains are unclear, they have been predicted to have important roles in redox or electron transfer reactions (Aravind, 2001). The DOMON domain is also predicted to bind with heme and/or sugars (Iyer et al., 2007). Biochemical studies showed that TcKnk extracted or released from cells has a very strong affinity for colloidal chitin (Chaudhari et al., 2011). Domain analysis of TcKnk-like proteins revealed a similar protein organization compared to TcKnk. TcKnk2 and TcKnk3 also have DM13 and DOMON domains, indicating that both of these TcKnk-like proteins may possess the ability to interact with chitin.

The widespread occurrence and retention of orthologs of *TcKnk2* and *TcKnk3* genes in other insect orders belonging to hemipteran, dipteran, lepidopteran and hymenopteran lineages indicates that duplication of the ancestral *Knk* gene from which these paralogs were derived must have occurred before the branching of these insect orders and that these *Knk-like* genes probably perform essential functions. While they are present in some arthropods, their absence in ticks, sea urchins and nematodes suggests that these are genes essential for some aspect of insect cuticle structure/function but not in all arthropods, chelicerates and nematodes which also have chitin in their exoskeleton

RNAi studies for *TcKnk2* and *TcKnk3* confirmed the importance of these genes for cuticle morphogenesis. *TcKnk2* dsRNA treatment led to ~55% molting arrest only at the pharate adult stage of development. Some of the surviving adults had abnormal wing phenotypes. *TcKnk3* also appears to be essential for survival and molting, as administration of any of several dsRNAs for this gene also yielded lethal phenotypes. The finding that the administered dsRNAs for *TcKnk2* and *TcKnk3* did not result in depletion of the essential *TcKnk* transcripts, and yet molting defects were observed, reveals specialized functions for TcKnk2 and TcKnk3-3' during cuticle morphogenesis, which are not redundant with those of TcKnk.

Unlike TcKnk, which is required for every molt, TcKnk2 and TcKnk3 have essential roles only during morphogenesis to the adult stage. Since TcKnk has been shown to be important both for the binding to chitin and for arranging the chitin into laminae in the larval body wall, adult elytral and adult abdominal body wall cuticles, the functions of TcKnk2 and TcKnk3 must be more specialized, perhaps involving specific tissues and/or cuticular structures such as the tracheae. It is known that substantial tracheal proliferation occurs during the pharate adult stage in *M. sexta* (Zimoch et al., 2005). We can see only a marginal reduction in total chitin content of whole animals at the pharate adult stage when treated with dsRNA for *TcKnk2* and *TcKnk3*. However, the requirement for the expression of these two *Knk*-like genes for survival and molting indicates that their function cannot be compensated by the expression of *TcKnk*.

The detection of three different alternatively spliced variants for *TcKnk3*, named *TcKnk3-FL*, *TcKnk3-5'* and *TcKnk3-3'*, necessitated the use of several exon-specific dsRNA treatments to selectively down-regulate one of these three mRNAs without affecting the other two. RNAi analysis for *TcKnk3* transcripts showed pharate adult molting arrest and lethality only when both the full length and *TcKnk3-3'* transcripts were depleted. On the other hand, a nearly complete loss of the *TcKnk3-5'* transcript along with the *TcKnk3-FL* transcript of this gene did not result in mortality or any visible phenotypes. The finding that *Tc-Knk3-5'* and *Tc-Knk3-3'* transcripts appear only when the full-length transcript is down-regulated raises the interesting possibility that these are utilized in compensatory mechanisms invoked only under conditions when the full-length protein is unavailable. The much higher levels of accumulation of the *TcKnk3-3'* transcript compared to *dsTcVer*-treated control when dsRNA for exon 5 is administered is consistent with such a compensating mechanism, as these insects are viable in the near complete absence of the full-length transcript and by implication the corresponding protein.

While TcKnk affects the total chitin content dramatically both at the pharate pupal and pharate adult stages (Chaudhari et al., 2011), the effects of RNAi for the other TcKnk-like genes are not significant at the pharate pupal stage, consistent with the absence of observable effects on molting or morphology at the larval-to-pupal molt. Even though there was some reduction in total chitin content after *TcKnk2* as well as *TcKnk3* RNAi at the pharate adult stage, we could not detect differences in chitin accumulation in the procuticle seen following *TcKnk* RNAi (Chaudhari et al., 2011). TEM analysis of body wall and tracheae of *TcKnk2* and *TcKnk3* dsRNA-treated pharate adult insects showed an amorphous procuticle and accumulation of

proteins at the tip of the taenidia, respectively. However, ultrastructural analysis of elytra showed normal laminar architecture of chitin in the procuticle following RNAi for *TcKnk2* and *TcKnk3*. Chitin content and TEM analysis revealed an important role for *TcKnk*-like genes in cuticular chitin maintenance and organization in some tissues like body wall denticles and trachea. A previous study with *D. melanogaster knk* mutants has revealed a role for Knk in embryonic tracheal tube expansion and tracheal lumen chitin microfibril assembly (Moussian et al., 2006). Accumulation of proteins in tracheae after *TcKnk2* and *TcKnk3* dsRNA treatment indicates a possible role for TcKnk-like proteins in influencing proper trafficking of proteins into tracheal lumen. Furthermore, dissected ovaries from beetles after *TcKnk2* RNAi showed the absence of developing oocytes in ovarioles, which gave a bead-like appearance to the ovaries. Lack of oocytes in *TcKnk2* knockout ovaries indicated an important role for *TcKnk2* in oocyte development and therefore maintenance of ovarian structure.

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## **Chapter 6 - Summary and Discussion**

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## General conclusions

Chitin, a homopolymer of  $\beta$  1, 4 linked N- acetylglucosamine subunits, is the major component of insect cuticle (Arakane et al., 2005). Chitin synthesis is catalyzed by a membrane-bound chitin synthase enzyme located at the apical plasma membrane of epithelial cells (Merzendorfer, 2006). Chitin chains are secreted into the extracellular space and are organized into chitin bundles. Several such bundles are then arranged to form chitin sheets. Chitin sheets are organized into laminae by an unknown mechanism(s) to give flexibility to the insect cuticle (Vincent and Wegst, 2004). Cuticular proteins from different insect species have been identified and predicted to bind with chitin and help in organization of the cuticle made up of chitin and a large assortment of proteins (Willis, 2010). A genetic screen for *D. melanogaster* embryos with cuticular defects has identified two genes named *knickkopf* (*knk*) and *retroactive* (*rtv*) along with *krotzkopf verkehrt* (*kkv*) (orthologue of *T. castaneum* *Chs-A*) (Ostrowski et al., 2002). *knk* mutant embryos showed broken head skeleton phenotype while *rtv* mutant embryos had a reversed polarity inside the eggshell. In addition to these phenotypes, both *knk* and *rtv* mutants showed “blimp” phenotypes like *kkv* mutants, indicating roles for *knickkopf* and *retroactive* in maintenance of cuticle integrity (Ostrowski et al., 2002). Further analysis of both these genes in cuticle development in *D. melanogaster* indicated essential role in tracheal tube expansion and tracheal luminal chitin filament assembly (Moussian et al., 2005; Moussian et al., 2006). Additionally, both *knk* and *rtv* were shown to be essential for procuticular chitin organization (Moussian et al., 2005; Moussian et al., 2006). However, the precise molecular mechanisms underlying their role(s) in organization of chitin have not been studied so far.

In order to grow and develop, insects must undergo a process of molting wherein fully developed and organized old cuticle is removed and replaced with a new one. The classical model of insect molting starts with separation of old cuticle from epithelial cells by the process of apolysis (Locke and Huie, 1979). The apolytic space created between old cuticle and epithelial cells is then filled with molting fluid containing chitinolytic and proteolytic enzymes (Passonneau and Williams, 1953). To avoid the direct contact between the new cuticle and molting fluid enzymes an envelope layer is formed (Locke, 2001). Simultaneously when old cuticle is being degraded by chitinases, new cuticle is synthesized. Once the old cuticle separation is nearly complete, insects undergo the process of ecdysis which involves a series of

characteristic contractions, during which the remnants of the old cuticle such as envelope, epicuticle and exocuticle are removed.

It has been believed for the last 50 years that an envelope layer, synthesized by epithelial cells prior to the secretion of new chitin-rich procuticular layers, acts as a physical barrier and protects the site of new chitin synthesis from coming in direct contact with chitinases (Locke, 2001). However, experimental evidence in support of this hypothesis has not been presented so far. Although, chitinase localization was tested in one study involving molting larvae of spruce budworm, its localization with respect to new developing procuticular chitin was not studied in detail (Zheng et al., 2003). In an attempt to understand localization of chitinases in molting insects, I localized chitinase-5 (TcCht-5) (the major molting fluid chitinase) in *T. castaneum* pharate adults (**Chapter 2**). The immunolocalization study not only detected TcCht-5 in the old cuticle, but surprisingly, but also in the newly synthesized procuticular chitin. In this dissertation I demonstrate for the first time that the chitinase co-localizes with newly synthesized procuticle. Although chitinases are localized in both old as well as new cuticle, it is interesting to observe how selectively only the old cuticle is degraded by chitinases during molting. In this thesis, **Chapter 2** focuses on the factors that help in protection of newly synthesized chitin from degradation by chitinases. As discussed earlier, Knk and Rtv have been shown to be important for cuticle organization in *D. melanogaster*. RNAi analysis in *T. castaneum* for *TcKnk*, *TcRtv* and *TcChs-A* resulted in molting arrest at each stage of development. Based on the phenotypic similarities among *TcKnk*, *TcRtv* and *TcChs-A* dsRNA-treated insects, we predicted that these genes influence the same pathway of cuticle biogenesis. Significant decreases in chitin content following dsRNA treatment for *TcKnk* and *TcRtv* confirmed an essential role for these genes in cuticular chitin maintenance.

Further biochemical analysis confirmed TcKnk to be a GPI anchored membrane-bound protein with the ability to bind to colloidal chitin *in vitro*. Immunolocalization studies indicated the presence of TcKnk on the apical side of the epithelial cells and inside the developing new procuticle. Like TcCht-5, TcKnk was found to be co-localized with newly synthesized procuticular chitin. Based on the observations that TcKnk protein co-localizes and binds with chitin, and that depletion of transcripts for *TcKnk* results in loss of chitin, we hypothesized an important role for TcKnk in maintenance of chitin in the newly developing procuticle by either affecting chitin synthesis or by controlling chitin degradation. In order to determine whether

TcKnk controls chitin synthesis by affecting TcChs-A protein localization, we immunolocalized TcChs-A and observed the cuticle under a confocal microscope. Confocal analysis indicated normal distribution of TcChs-A protein in insects treated with *TcKnk* dsRNA indicating no role for TcKnk in proper trafficking of TcChs-A and/or synthesis of chitin. However, injections of dsRNAs for *TcKnk* and *TcCht-5* together restored the chitin levels to normal indicating a role for TcKnk in protecting the newly synthesized chitin from degradation by chitinases. This discovery casts strong doubt on the long-held theory that an impermeable envelope is sufficient to ensure this selective susceptibility of old versus new cuticle to molting enzymes. Our finding that Knk levels are depleted in the old cuticle, which is quite susceptible to degradation by chitinases, when the chitin in the procuticle of the newly forming cuticle is quite stable due to the presence of high levels of Knk protein, is consistent with our hypothesis that Knk protects chitin either by binding to it or by organizing it into a chitinase-resistant structure.

Our study with insects treated with nikkomycin (a competitive inhibitor of chitin synthase-A) indicated mislocalization of TcKnk from procuticle to within the epithelial cells, confirming the importance of presence of extracellular chitin for proper trafficking of TcKnk. Like nikkomycin treatment, *TcRtv* dsRNA injections lead to mislocalization of TcKnk from the procuticle to within the epithelial cells (**Chapter 3**). The depletion of chitin observed after *TcRtv* RNAi was predicted to be a secondary effect due to mislocalization of TcKnk, as chitin in the procuticle was not protected from chitinases by the presence of Knk. As hypothesized, co-injection of dsRNAs for *TcRtv* along with *TcCht-5* rescued the chitin depletion phenotype, confirming an indirect role for TcRtv in protecting chitin from chitinases. Despite complete recovery of chitin levels in insects treated with dsRNAs for both *TcRtv* and *TcCht-5*, TcKnk was still mislocalized inside the cells indicating importance for TcRtv in proper trafficking of TcKnk to the procuticle.

In addition to the post-embryonic studies, we have found roles for TcKnk and TcRtv during serosal cuticle secretion during embryonic development of *T. castaneum* (**Chapter 4**). Our work shows that both TcKnk and TcRtv are required for serosal cuticle development and its laminar organization by maintaining normal levels of chitin in embryos. In this dissertation, I demonstrate the presence and expression of additional *TcKnk*-like genes in different insect species for the first time. I have identified two *TcKnk* like genes in *T. castaneum* genome and named them as *TcKnk2* and *TcKnk3* (**Chapter 5**). Orthologs of *T. castaneum* *Knk*-like genes are

identified in all insect species with fully sequenced genomes. Both *TcKnk2* and *TcKnk3* dsRNA-treated insects show molting arrest only at the pharate adult stage of *Tribolium* development. Transmission electron microscopic analysis of *T. castaneum* pharate adult elytra, body wall and tracheal tissues indicated no differences in the laminar architecture of the elytral cuticle, but body wall denticles and trachea were defective. It is important to note that *TcKnk*-like genes affect only the presence or organization of non-laminar chitin in the denticles and trachea, indicating their probable function in protecting non-laminar chitin from chitinase degradation.

## **Future Directions**

Although the exact mechanism of protection of chitin by TcKnk is still a mystery, several possibilities exist. It is possible that the direct binding with TcKnk masks chitin and protects it from degradation by chitinases. Alternatively, TcKnk might inhibit chitinases by direct protein-protein interaction and thus provide protection. Given that only the old cuticle needs to be degraded during molting, it may be argued that the protective effect against the major chitinases is limited only to the new cuticle. *T. castaneum* pharate adult elytral sections indicate localization of TcKnk predominantly in the new cuticle. Absence of TcKnk in the old cuticle may account for its susceptibility to chitinases. The mechanism through which TcKnk disappears from the old cuticle is still unknown. It can be predicted that the proteolytic enzymes from molting fluid degrade TcKnk from the old cuticle and make chitin more accessible to chitinase action. It is interesting to point out that RNAi of a chymotrypsin-like protein, which is present in cast cuticles, results in molting defects similar to those seen with RNAi for TcKnk and TcRtv (Broehan et al., 2010). The role of such chymotrypsin-like proteins in degradation of TcKnk, if any, needs to be analyzed in future. It will be interesting to localize the proteases in the old versus the new cuticle as well.

Trafficking of TcKnk from cytoplasmic vesicles to an extracellular location within the procuticle is dependent on extracellular chitin and presence of TcRtv protein inside the cells. Our work has uncovered a major pathway involved in cuticular chitin maintenance wherein extracellular chitin intermediates synthesized by Chs-A and presence of TcRtv inside the cells, trigger the accretion of Knk to the growing chitinous matrix of the procuticle whereupon it may catalyze chitin organization and render protection from chitinolytic enzymes. The exact mechanism by which TcRtv facilitates the proper trafficking of TcKnk is still unknown. Like *D.*

*melanogaster* Rtv (Moussian et al., 2005), bioinformatics analysis for TcRtv protein indicated the presence of aromatic residues that can potentially bind with chitin. From the available data, it can be predicted that chitin interacts with TcRtv and sends a signal to TcKnk for proper trafficking to the procuticle. Binding of Rtv with chitin needs to be tested in future. Alternatively, Rtv might interact with Knk by direct protein-protein interaction ensuring proper localization of latter protein. Once transported to the plasma membrane, TcKnk is cleaved and deposited in the developing new cuticle. Proteins involved in cleavage of the membrane-bound GPI-anchored TcKnk, such as endogenous phospholipase-C or phospholipase D, or other enzymes, need to be investigated. Once released into the growing chitinous matrix, TcKnk protects the newly synthesized chitin from chitinases. Because Knk and Rtv are unique conserved proteins crucial for chitin maintenance in all insect pests, it may represent an excellent target for biocontrol agents.



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**In**

### **CHAPTER 2**

## **The Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton**

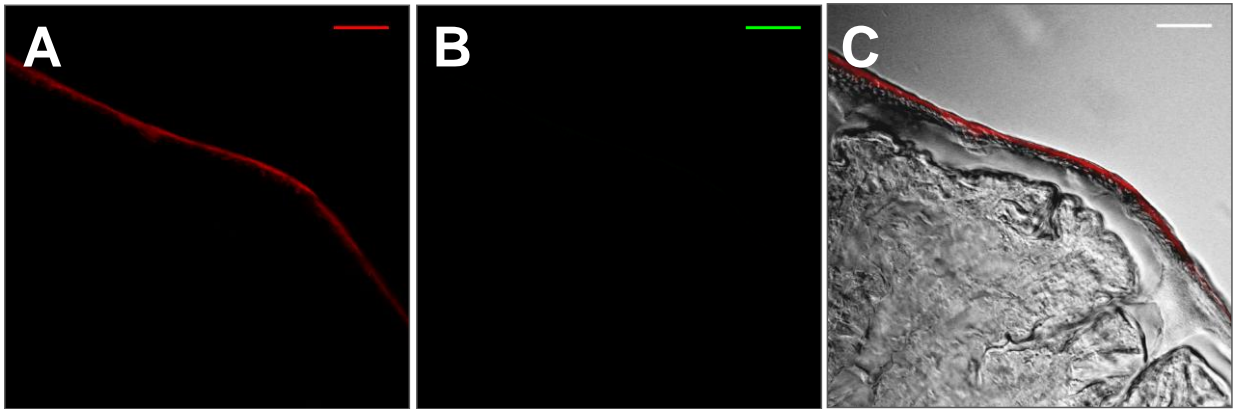
Chaudhari S. S., Arakane Y., Specht C. A., Moussian B., Boyle D. L., Park Y., Kramer K. J.,  
Beeman R. W., Muthukrishnan S. (2011).

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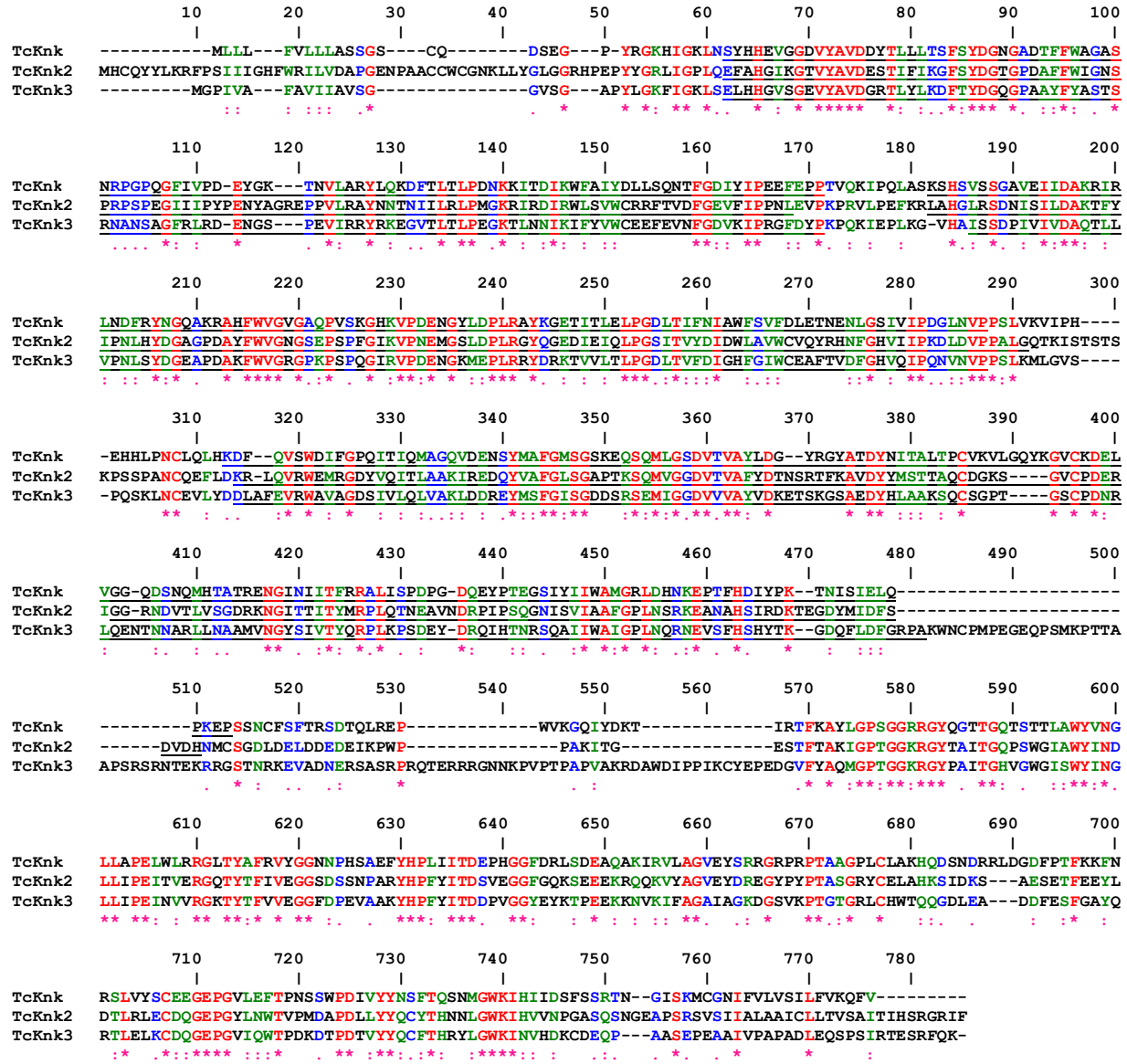
## Appendix B - Immunohistochemistry controls



### **Immunohistochemistry controls for *T. castaneum* cryosections confocal analysis.**

*T. castaneum* cryosections were analyzed under LSM META 510 laser scanning confocal microscope without any antibody for autofluorescence. (A) Samples showed red autofluorescence for the cuticle after excitation at 543 nm. (B) No autofluorescence was detected for the samples at 488 nm excitation. (C) BL images showing the merged images for cuticle and red autofluorescence. Scale bar= 10  $\mu$ m.

# Appendix C - Amino acid alignment for TcKnk, TcKnk2 and TcKnk3



## Amino acid sequence alignment of TcKnk, TcKnk2 and TcKnk3.

Shading of amino acid residues in different colors indicate identity and similarity. Underlines indicate different DM13 and DOMON domains.