



Both UDP *N*-acetylglucosamine pyrophosphorylases of *Tribolium castaneum* are critical for molting, survival and fecundity

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ABSTRACT

A bioinformatics search of the genome of the red flour beetle, *Tribolium castaneum*, resulted in the identification of two genes encoding proteins closely related to UDP-*N*-acetylglucosamine pyrophosphorylases (UAPs), which provide the activated precursor, UDP-*N*-acetylglucosamine, for the synthesis of chitin, glycoproteins and glycosylphosphoinositide (GPI) anchors of some membrane proteins as well as for the modification of other substrates. This is in contrast to other arthropods whose genomes have been completely sequenced, all of which have only a single copy of this gene. The two *T. castaneum* UAP genes, *TcUAP1* and *TcUAP2*, share both nucleotide and amino acid sequence identities of about 60%. RT-PCR analysis revealed that the two genes differ in their developmental and tissue-specific patterns of expression. RNA interference (RNAi) indicated roles for *TcUAP1* and *TcUAP2* at the molt and intermolt stages, respectively: RNAi for *TcUAP1* resulted in specific arrest at the larval-larval, larval-pupal or pupal-adult molts, depending on time of injection of double-stranded RNAs, whereas RNAi for *TcUAP2* prevented larval growth or resulted in pupal paralysis. Analysis of elytral cuticle indicated loss of structural integrity and chitin staining after RNAi for *TcUAP1*, but not after RNAi for *TcUAP2*. Loss of peritrophic matrix (PM)-associated chitin was also observed following RNAi for *TcUAP1*, but not after RNAi for *TcUAP2*. Down-regulation of transcripts for either *TcUAP* gene at the mature adult stage resulted in cessation of oviposition in females, as well as fat body depletion and eventual death in both sexes. These results demonstrate that both *TcUAP* genes are critical for beetle development and survival, but that only *TcUAP1* is clearly associated with synthesis of cuticular or PM chitin. However, both of these genes appear to have additional critical role(s) unrelated to chitin synthesis, presumably in the glycosylation of proteins and/or secondary metabolites.

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1. Introduction

Chitin is an essential component of the exoskeleton and the peritrophic matrix (PM) of insects. It is a linear homopolymer of the sugar, *N*-acetylglucosamine, connected by β -1,4-linkages. The biosynthetic pathway of this sugar begins with the glycolytic

Abbreviations: *Tc*, *Tribolium castaneum*; UAP, UDP-*N*-acetylglucosamine pyrophosphorylase; UGP, UDP-glucose pyrophosphorylase; FITC-CBD, Fluorescein isothiocyanate-conjugated chitin-binding-domain; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GPI, Glycosylphosphatidylinositol; PM, Peritrophic matrix; CHS, Chitin synthase; RT-PCR, Reverse transcription-polymerase chain reaction; *Ver*, *vermilion*; gDNA, genomic DNA; CDS, coding sequence.

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intermediate fructose-6-phosphate and involves the amination of its 2-keto group, followed by acetylation of the 2-amino group and isomerization of the phosphorylated sugar to yield *N*-acetylglucosamine-1-phosphate. This sugar phosphate is further activated by the enzyme UDP-*N*-acetylglucosamine pyrophosphorylase (UAP), which utilizes the high-energy pyrophosphate bonds of the co-substrate UTP to yield the nucleotide-sugar, UDP-*N*-acetylglucosamine (UDP-GlcNAc). The latter is the basic building block for the biosynthesis of chitin (Kramer and Muthukrishnan, 2005; Merzendorfer, 2006; Kato et al., 2006; Moussian, 2008). UAP is also critical for the glycosylation of proteins, sphingolipids and secondary metabolites with *N*-acetylglucosamine (GlcNAc) or glycosylphosphatidylinositol (GPI) anchors (in the case of some membrane-bound proteins), or for conjugation of 7- β -hydroxylated bile acids (Marschall et al., 1992).

The enzyme UAP is widely distributed in nature and is closely related to the enzyme UDP-glucose pyrophosphorylase (UGP), which makes UDP-glucose for the synthesis of glycogen or for glycosylation of proteins or secondary metabolites. Some UAPs, perhaps all, can accept both GlcNAc-1-P and *N*-acetylgalactosamine (GalNAc)-1-P as the substrate, although the former is usually the preferred substrate (Szumilo et al., 1996).

UAP is essential for insect survival, as shown by studies on *Drosophila melanogaster* mutants of the single gene that encodes UAP in that species. This gene, variously called *mummy*, *cabrio* or *cystic*, is required for cuticle organization, tracheal tube morphogenesis and glycosylation of proteins (Araujo et al., 2005; Tonning et al., 2006; Schimmelpfeng et al., 2006). Mutants of this gene exhibit defects in tracheal tubule development, dorsal closure, central nervous system fasciculation and eye development. Some of these defects are due to reduced chitin content of the epidermal cuticle or of the taenidial folds of tracheal tubes, while others such as fasciculation defects and eye development abnormalities are likely to be due to defective glycosylation of glycoproteins.

Previously, using RNA interference (RNAi) experiments, we showed that down-regulation of transcripts for chitin synthases (CHSs) resulted in reduction in chitin content of the cuticle and the gut-associated PM in larvae and adults of *Tribolium castaneum* (Arakane et al., 2005, 2008). Furthermore, we demonstrated that there is specialization in the functions of the two genes encoding CHSs, namely that *CHS-A* (previously referred to as *CHS-1*) is required exclusively for synthesis of chitin in the cuticle secreted by ectodermally-derived epidermal cells, whereas *CHS-B* (previously referred to as *CHS-2*) functions only in synthesis of chitin in the PM secreted by the endodermally-derived midgut epithelium. It was of interest to determine whether there is a corresponding specialization in the functions of genes that encode UAPs, which supply the substrates for CHSs and other glycosyltransferases in different tissues. In this paper, we describe the identification of two genes encoding UAPs in *T. castaneum* and their patterns of expression during development and in different tissues. We also present evidence that only one UAP gene, *TcUAP1*, is essential for synthesis of chitin in cuticular structures and PM during development, but that both genes affect viability in the adult stage, possibly via mechanisms involving glycosylation of proteins or other substrates.

2. Materials and methods

2.1. Insect cultures

The GA-1 strain of *T. castaneum* was used in 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).

2.2. Identification of UAP genes in the *T. castaneum* genome database

A TBLASTN search of the *T. castaneum* genome was carried out at Beetlebase (<http://www.bioinformatics.ksu.edu/Beetlebase>) using the amino acid sequence of *D. melanogaster* UAP as the query. This resulted in the identification of two genes that we have named *TcUAP1* and *TcUAP2*. A second set of searches was done using the amino acid sequences of the two *T. castaneum* UAP proteins as queries in an attempt to identify additional genes encoding proteins related to UAPs.

2.3. Cloning and sequencing of cDNAs encoding UAPs

Gene-specific primers were designed to flank the predicted coding sequences (CDSs) of the two *TcUAP* genes. The *TcUAP1* forward primer 5'-ATGTCAGAAATCGACGAAATCAG-3' and the reverse primer 5'-TTACGAACAGGTGAGTTCTTCGTC-3' were used to amplify its 1446 bp CDS. The *TcUAP2* forward primer 5'-GACAC-GATGGCAAATTCGATG-3' and the reverse primer 5'-TTAAGCCCTCCCACCATTAATAC-3' were used to amplify its 1449 bp CDS. Using cDNA templates prepared from total RNA isolated from insects at different developmental stages and the two pairs of gene-specific primers for *TcUAP1* and *TcUAP2*, we amplified two DNA fragments with the expected sizes and cloned them in the pGEM-T vector (Promega, Madison, WI). Sequencing of these cDNA clones was carried out at the DNA sequencing facility at Kansas State University. The accession numbers of the two *TcUAP1* and *TcUAP2* clones are GU228846 and GU228847, respectively.

2.4. Determination of expression profiles of *TcUAP* genes

The RNeasy Mini kit (Qiagen, Valencia, CA) was used to isolate total RNA from embryos, larvae, pharate pupae, pupae and adults according to the manufacturer's instructions. Sex cannot be determined in larvae. For pupae and adults, approximately equal numbers of males and females were used for each RNA preparation, unless otherwise indicated. For determination of tissue-specificity of expression, total RNA was also isolated from a variety of larval, pupal and adult tissues. The midguts from 10 insects were divided into anterior, middle and posterior thirds, the segments from each region being pooled for RNA isolation. The total RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX, 2 U/μl) for 20 min at 37 °C to remove genomic DNA (gDNA) contamination, since fragments amplified from gDNA templates would be indistinguishable from those amplified from cDNA templates of these intronless genes (see below). The Superscript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize first-strand cDNA according to the manufacturer's instructions. Oligo-(dT)₂₀ was used as primer for reverse transcription using 1 μg of total RNA as template. The following pairs of primers were used to analyze the tissue-specificity of expression of each *TcUAP* gene: 5'-TGCTCGAGCAGCTCAAATCC-3' and 5'-GAACTTCTCCGTGGCTTCG-3' for *TcUAP1*, and 5'-TTGGATTTGGACGCCAGTGC-3' and 5'-CTAAAACACCGTTCACGTGC-3' for *TcUAP2*. *TcRPS6* (ribosomal protein S6) was amplified to serve as an internal loading control for RT-PCR (Arakane et al., 2010).

2.5. Phylogenetic analysis of UAP and UGP proteins

Multiple sequence alignments of the conserved domain cd04193 of eukaryotic UAP proteins (approximately 70% of each complete protein sequence) and the corresponding region of the related UGP protein from *T. castaneum* were carried out using the ClustalW software from (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl) prior to phylogenetic analysis. MEGA 4.0 (Tamura et al., 2007) was utilized to construct the consensus phylogenetic tree, using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). To evaluate the branch strength of the phylogenetic tree, bootstrap analysis of 500 replications was performed.

2.6. RNA interference studies

Three regions of *TcUAP1* and *TcUAP2* cDNAs that were most divergent in their nucleotide sequences were chosen as target regions for RNAi (Supplementary table 1). Pairs of forward and

reverse primers corresponding to these regions with additional T7 promoter sequences at the 5'-ends were synthesized and used for the preparation of double-stranded RNAs (dsRNAs) using an Ampliscribe T7-Flash Transcription Kit (Epicentre Technologies, Madison, WI) as described previously (Arakane et al., 2005). A control dsRNA for the *vermilion* gene (*TcVer*), which affects eye color (Lorenzen et al., 2002), served as a positive control for effectiveness of RNAi and as a negative control for monitoring non-specific effects of the RNAi protocol. The purified dsRNAs were injected into larvae, pharate pupae or mature adults (200 ng per insect, $n = 30$). After 3 d, total RNA preparations were made from pools of five insects for measuring transcript levels by RT-PCR using gene-specific primer-pairs. The remaining insects were observed daily for any visible abnormalities and mortality.

2.7. Analysis of elytra for integrity and presence of chitin

One-d-old pupae were injected with dsRNAs for *TcUAP1*, *TcUAP2* or *TcVer* as described in Section 2.6. The elytra were dissected from 5-d-old pupae and incubated with 10 M NaOH at 95 °C for 5 h to solubilize most of the cuticular proteins. The remaining elytral scaffold was observed under a light microscope for retention/loss of structural integrity of the chitinous cuticular matrix and then stained with fluorescein isothiocyanate-conjugated chitin-binding-domain probe (FITC-CBD, New England Biolabs, Ipswich, MA) (Arakane et al., 2005). The fluorescence was observed using a Leica MZ FLIII fluorescence stereomicroscope equipped with the following filter set: excitation = 480/40 nm, barrier = 510 nm.

2.8. Analysis of peritrophic matrix by chitin staining with FITC-CBD

Larvae in penultimate or earlier larval instars were injected with dsRNAs for *TcUAP1*, *TcUAP2*, *TcCHS-B* or *TcVer* (200 ng/larva, $n = 20$). Three days after injection, guts were isolated from treated larvae and PM-associated chitin was stained with FITC-CBD. The stained guts were photographed as described in Section 2.7.

3. Results

3.1. Identification of two genes encoding UAPs in the *T. castaneum* genome

BLASTP and TBLASTN searches of the *T. castaneum* genome resulted in the identification of two genes encoding closely related proteins similar to *D. melanogaster* UAP. These genes were named *TcUAP1* and *TcUAP2*. *TcUAP1* maps to linkage group 1 (=X), position 52.7 cM, while *TcUAP2* is located on linkage group 8 at position 28.0. A second set of searches using the two *T. castaneum* UAP protein sequences as queries failed to identify additional genes encoding proteins related to UAPs in the *T. castaneum* genome.

Using forward primers that included the presumed start codon of each CDS and reverse primers whose 5'-ends were complementary to the TAA stop codons, and using total RNA prepared from pupal stages as template, we were able to amplify two cDNA fragments with sizes of about 1.4 kbp by RT-PCR. Comparisons of the sequences of the cDNA clones with the genome sequences indicated that these two *TcUAP* genes had no introns. The two sequences showed approximately 60% identity at both the nucleotide and amino acid levels, indicating that the two genes/proteins are closely related. They share about 58 and 53% identity, respectively, with the nucleotide and encoded amino acid sequences of the single *D. melanogaster* UAP gene. Fig. 1 shows an alignment of the two *T. castaneum* proteins. *TcUAP1* is 481 amino acids long, while *TcUAP2* is longer by one amino acid. Neither protein is predicted to have a leader peptide or a trans-membrane segment or other organelle-targeting sequences, indicating that these are likely to be cytosolic enzymes.

3.2. Phylogenetic analysis of arthropod UAPs and UGPs

The UAPs are a subclass of nucleotide pyrophosphorylases involved in the supply of activated precursors of sugars needed for the biosynthesis of sugar polymers, glycoproteins or other glycoside conjugates. They are most closely related to UDP-glucose



Fig. 1. Deduced amino acid sequence alignment of TcUAP1 and TcUAP2 using ClustalW software. The two proteins share 61% amino acid sequence identity. Symbols below the aligned amino acid sequences indicate identical (*), highly conserved (:), and conserved residues (.). Boxes indicate residues important for substrate binding. Both enzymes have a cysteine at position 330, but in human UAP and *C. elegans* UAP, this residue is an alanine.

pyrophosphorylases (UGPs), which catalyze the synthesis of UDP-glucose needed for the production of glycogen, glycoproteins or other glycoside conjugates. Representative arthropods from dipteran, lepidopteran, hymenopteran, hemipteran, phthirapteran, tick and crustacean lineages each have only one gene encoding a UAP. Among the arthropods whose genomes have been completely sequenced, *T. castaneum* is unique in having two genes encoding UAPs. Fig. 2 is a phylogenetic tree indicating the evolutionary relationship between various arthropod, nematode, mammalian and yeast UAPs, as well as two representative insect UGPs. As expected, the two *T. castaneum* UAPs are tightly clustered in the phylogenetic tree. The UAPs constitute one branch of the evolutionary tree shown in Fig. 2, while the UGPs form a separate branch. UGP is a single-copy gene in all arthropod species examined, including *T. castaneum* and *D. melanogaster* (Fig. 2 and data not shown).

3.3. Expression profiles of *TcUAP* genes during development

To determine whether there are any differences in the expression profiles of the two *TcUAP* genes during *T. castaneum* development, we analyzed RNA preparations from embryos, larvae (penultimate and last-instar), pharate pupae, pupae and adults (0–1-d-old and 4–5-wk-old) for transcripts for these two *UAP* genes by RT-PCR using gene-specific primer-pairs. Transcripts for both *UAP* genes were detected at all developmental stages including trace amounts in the embryonic stages (Fig. 3A).

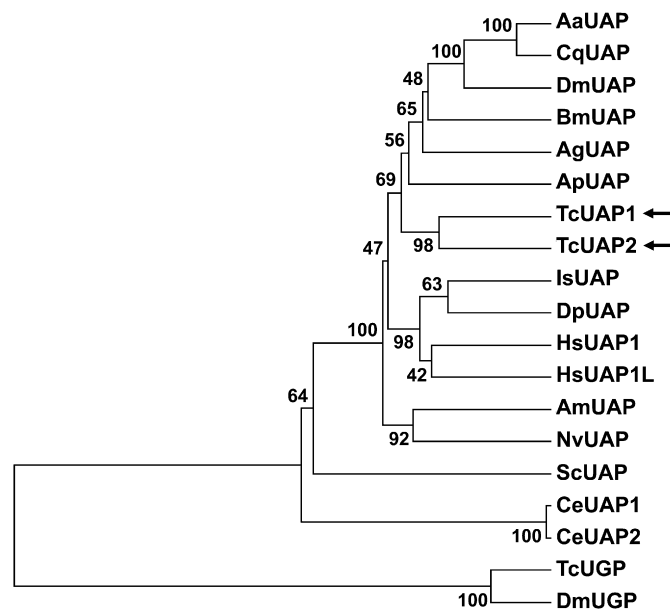


Fig. 2. Phylogenetic analysis of insect UAPs. MEGA 4.0 was used to construct the phylogenetic tree using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Tamura et al., 2007). Bootstrap analyses from 500 replications are shown by each branch. Arrows indicate *T. castaneum* UAP proteins. UAP: UDP-N-acetylglucosamine pyrophosphorylase. UGP, UDP-glucose pyrophosphorylase. AaUAP, *Aedes aegypti* (EAT47260); CqUAP, *Culex quinquefasciatus* (EDS38218); DmUAP, *Drosophila melanogaster* (NP_609032); BmUAP, *Bombyx mori* (BGIBMGA001609-PA); AgUAP, *Anopheles gambiae* (XP_317600); ApUAP, *Acyrtosiphon pisum* (XP_001944680); IsUAP, *Ixodes scapularis* (EEC12000); DpUAP, *Daphnia pulex* (NCBI_GNO_212930, wFleaBase); HsUAP1, *Homo sapiens* (NP_003106, isoform AGX2); HsUAP1L, *Homo sapiens* (AAI06036, UAP1L1, isoform 1) AmUAP; *Apis mellifera* (XP_624349); NvUAP, *Nasonia vitripennis* (XP_001602623); ScUAP, *Saccharomyces cerevisiae* (NP_010180.1); CeUAP1, *Caenorhabditis elegans* (NP_497777); CeUAP2, *Caenorhabditis elegans* (NP_500511.2); TcUGP, *Tribolium castaneum* (GLEAN_09819); DmUGP, *Drosophila melanogaster* (NP_648300).

3.4. Tissue-specificity of expression of *UAP* genes

When total RNA preparations from different tissues from the larval stages were analyzed, transcripts for *TcUAP1* were widely distributed in larval tissues (Fig. 3B). Trace amounts of *TcUAP2* transcripts were detected in the larval midgut, hindgut and fat body, but not in the integument (Fig. 3B). Transcripts from a predominantly cuticle-forming tissue, the adult elytron, were also analyzed during its morphogenesis. In this tissue, *TcUAP1* transcripts showed maximum abundance on day 0 of adult eclosion, with lower but detectable levels from the late pupal (two days before adult eclosion) through 5-day-old adult stages. The expression patterns of this gene, which appears to supply the activated UDP-GlcNAc precursor for chitin synthesis, closely paralleled that of *TcCHS-A*, which catalyzes the polymerization of this precursor into chitin in this tissue (Fig. 3C). In contrast, transcript levels for *TcUAP2* remained nearly constant during this period. Both male and female reproductive tissues were found to contain transcripts for both *UAP* genes in adults (Fig. 3D), but unlike the case in larvae, adult fat body showed little or no expression of either gene (unpublished data).

3.5. RNAi at larval, pupal and adult stages

RNA interference (RNAi) experiments were carried out using dsRNAs targeting three different regions of each of the two *TcUAP* genes (Supplementary table 1) to determine the role of each UAP in *T. castaneum* development and molting. dsRNAs were injected into young larvae (penultimate larval instar or younger), last-instar larvae, pharate pupae and 4–5-wk-old adults to assess the effects of RNAi on larval-larval, larval-pupal and pupal-adult molts as well as survival and fecundity. To ensure that the down-regulation of transcripts was specific for the targeted *TcUAP* gene, total RNA was isolated from these insects 3 days after dsRNA administration and transcript abundance for each *TcUAP* gene was analyzed by RT-PCR. As shown in Fig. 4A and B for young larvae, only the targeted *TcUAP* transcript was down-regulated, with no significant decrease in the levels of transcripts for the other (non-target) *TcUAP* gene. Similar results were seen at other stages and with the other two dsRNAs for each *TcUAP* gene (data not shown).

The phenotypes of insects following RNAi for *TcUAP1* or *TcUAP2* genes are shown in Fig. 5. Young larvae (penultimate instar or earlier larval stages) injected with dsRNA for *TcUAP1* (*dsTcUAP1*) failed to complete the larval-larval molt. Apolysis and slippage of the old larval cuticle were observed (top row, left panel), but all of the larvae were trapped in the old cuticle. In many cases, melanization of lateral tracheae was observed, as has been reported previously following RNAi for *TcCHS-A* (Arakane et al., 2005). This appears to be a consequence of the wound response that is expected when the weakened (chitin-deficient) new cuticle and epidermis are damaged during separation from the old tracheal cuticle. When injections of *dsTcUAP1* were made in the last larval instar, developmental arrest and death occurred during the larval-pupal molt when the insects became entrapped in their larval cuticles. The dorsal split was typically initiated and the new pupal cuticle was clearly visible, but the insects were unable to shed the old larval cuticle (top row, second panel from the left). When *dsTcUAP1* was injected into pharate pupae, the insects molted normally to the pupal stage, but subsequently failed to complete the adult molt and died as pharate adults entrapped in the pupal exuvium (top row, third panel from the left).

The consequences of RNAi for *TcUAP2* showed some similarities to, but also distinct differences from, those for *TcUAP1*. Unlike the case with *TcUAP1*, injections of *dsTcUAP2* at young larval stages always resulted in developmental arrest and size shrinkage prior to initiation of the subsequent larval-larval molt (Fig. 5, second row,

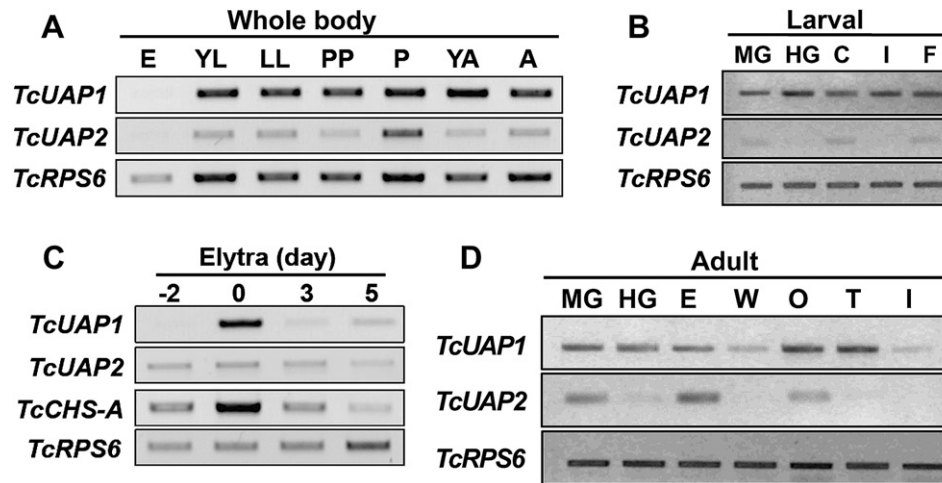


Fig. 3. Analysis of stage- and tissue-specific expression of *TcUAP1* and *TcUAP2* by RT-PCR. (A) Developmental expression profiles of *TcUAP1* and *TcUAP2*. cDNAs were prepared from total RNA extracted from whole beetles ($n = 5$, except for the embryonic stage) at several developmental stages including E, embryos; YL, young larvae (penultimate instar or younger); LL, last-instar larvae; PP, pharate pupae; P, pupae; YA, young adults (0–1-d post-eclosion); A, Adults (4–5 wks post-eclosion). (B) Expression of *TcUAP1* and *TcUAP2* in the larval midgut, hindgut, carcass, fat body and integument (MG, HG, C, I and F, respectively). “Carcass” in panel B refers to the entire body exclusive of gut, i.e., it includes fat body, integument and muscle. (C) Expression of *TcUAP1* and *TcUAP2* in the cuticle-forming tissue, adult elytra. Total RNA was isolated from elytra dissected from 3-d-old pupae (-2) (2 days before adult eclosion) and 0-, 3- and 5-d-old adults ($n = 10$). The expression pattern of *TcCHS-A*, that contributes exclusively to cuticular chitin (Arakane et al., 2005, 2008), was also analyzed. (D) Expression of *TcUAP1* and *TcUAP2* in various tissues from 4–5-wk-old adults. E, elytra; W, wings; O, ovaries; T, testes; I, abdominal integument. Other abbreviations as in panel (B). *T. castaneum* ribosomal protein S6 (*TcRps6*) was used as internal control for RT-PCR. Results are from 28 and 24 cycles of RT-PCR for *TcUAPs* and *TcRPS6*, respectively.

left panel, $n = 20$). When last-instar larvae were injected with dsRNA for *TcUAP2* (*dsTcUAP2*), the insects pupated normally (also unlike *TcUAP1*). However, these pupae suffered developmental arrest at the pharate adult stage, and all of the insects died without completing pupal-adult development (second row, middle panel). Similar results were obtained when pharate pupae were injected with *dsTcUAP2*, with all ($n = 20$) insects dying at the pharate adult stage. Although insects treated with *dsTcUAP2* as either last-instar larvae or pharate pupae showed the same terminal phenotype as those treated with *dsTcUAP1* in the pharate pupal stage, there was a striking difference in the behavior of the affected pupae. After *dsTcUAP1* treatment, pupae retained normal muscular activity and responded to touch by vigorous wiggling or writhing. In contrast, pupae developing after *dsTcUAP2* treatment appeared to be almost paralyzed and were relatively nonresponsive to touch.

The effect of RNAi at the adult stage was of interest because both *TcUAP* genes continue to be expressed throughout the adult stage in a variety of tissues (Fig. 3). When 4–5-wk-old adults of either sex were injected with dsRNA for either of the *TcUAP* genes, mortality was observed (preceded in females by cessation of oviposition), beginning a few days after injection and progressing over a several-week period (Fig. 5A). When the moribund animals were observed under a light microscope after removal of elytra and hindwings, depletion of fat body or autophagy was evident, revealing the

ventral abdominal segments when viewed from the dorsal side in both sets of animals (arrows in Fig. 5B middle and right panels). In contrast, larvae injected with a control dsRNA (dsRNA for *vermillion*, *dsTcVer*) underwent normal development, and the resulting adults had normal fertility and longevity, and had normal fat body, which masked the pigmented ventral segments when viewed from the dorsal side (Fig. 5B, left panel).

3.6. Analysis of elytral chitin content after RNAi

In a separate experiment, 1-d-old pupae injected with *dsTcUAP1*, *dsTcUAP2* or *dsTcCHS-A* were compared with controls (*dsTcVer*-injected) to study the effect of down-regulation of transcripts of these genes on the integrity of a chitin-containing structure, the adult elytron, which could be easily isolated from the rest of the body at the pharate adult stage. Light microscopic examination of elytra did not reveal any gross alterations in the integrity of elytra following any of the dsRNA treatments (Fig. 6, top row). After removal of cuticular proteins by NaOH treatment, the remaining chitinous matrix was intact in insects injected with *dsTcUAP2*. In contrast, elytra from insects injected with *dsTcUAP1* or *dsTcCHS-A* had essentially dissolved following removal of cuticular proteins by NaOH treatment (Fig. 6, second row). Only a few small fragments of the elytra were left behind, indicating loss of structural integrity or a nearly complete absence of the elytral cuticular matrix.

FITC-CBD staining of NaOH-treated elytra indicated that there was no detectable loss of chitin staining following RNAi for *TcUAP2*. Chitin staining was indistinguishable from that of *dsTcVer*-treated controls (Fig. 6, bottom panels). *TcCHS-A* has been shown to be exclusively responsible for cuticular chitin synthesis (Arakane et al., 2005). It thus appears that the major contributor to the synthesis of UDP-GlcNAc needed for cuticular matrix chitin synthesis in this tissue is *TcUAP1* and not *TcUAP2*.

3.7. RNAi for *TcUAP1*, but not for *TcUAP2*, results in loss of chitin staining of PM

To assess the contributions of *TcUAP1* and *TcUAP2* in providing precursors for the synthesis of PM-associated chitin in *T. castaneum*,

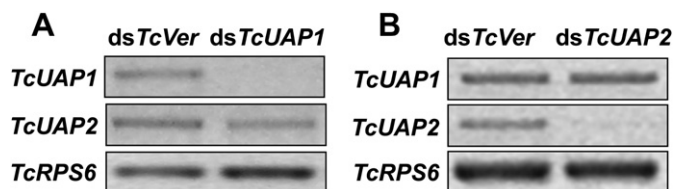


Fig. 4. Specific down-regulation of *TcUAP1* and *TcUAP2* transcripts by RNAi. dsRNAs (200 ng per insect) for *TcUAP1* (*dsTcUAP1*) and *TcUAP2* (*dsTcUAP2*) were injected into young larvae (penultimate instar or younger, $n = 20$). Three days after injection, total RNA was extracted from whole middle-instar stage larvae ($n = 3$) and used for cDNA synthesis. Transcript levels of *TcUAP1* and *TcUAP2* were detected as described in legend to Fig. 3. *T. castaneum* ribosomal protein S6 (*TcRPS6*) was used as internal loading control. dsRNA for *T. castaneum* *vermillion* (*dsTcVer*) was injected as a control.

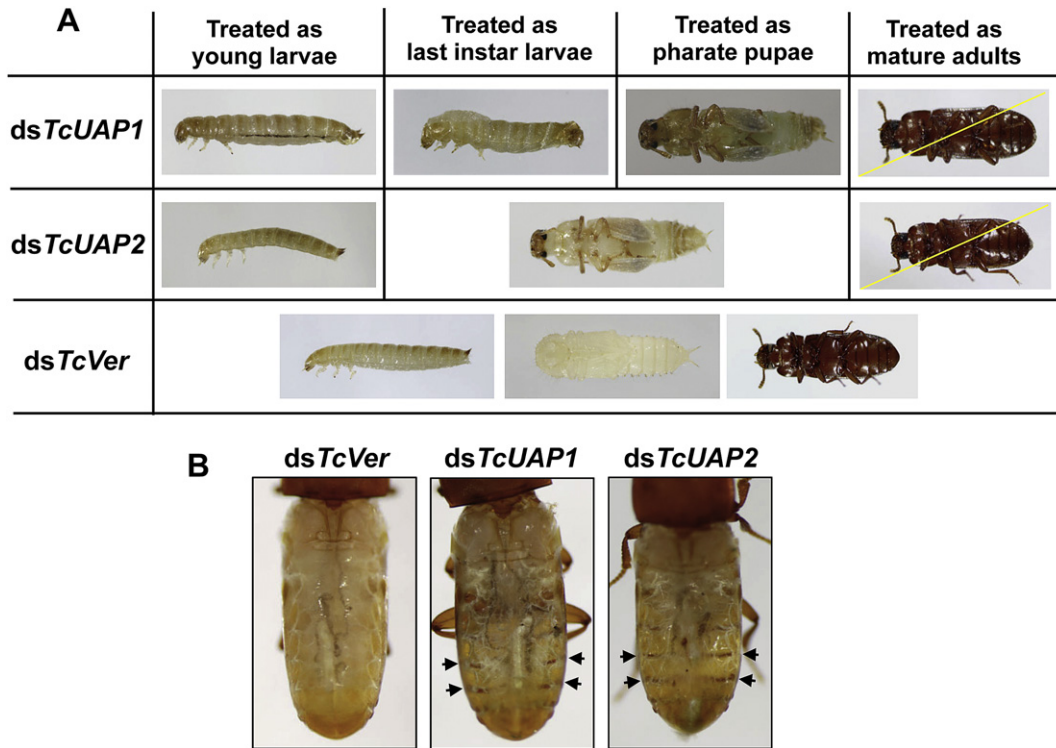


Fig. 5. Lethal phenotypes produced by injection of dsRNAs for *TcUAP1* and *TcUAP2*. (A) dsRNAs for *TcUAP1* (*dsTcUAP1*) and *TcUAP2* (*dsTcUAP2*) were injected into young larvae, last-instar larvae, pharate pupae and mature (4–5-wk-old) adults. Yellow lines indicate moribund adults. (B) Indicated dsRNAs were injected into 4–5-wk-old adults and the phenotypes were observed after 2 weeks. Little or no fat body was observed in the adults treated with either *dsTcUAP1* or *dsTcUAP2*. The arrows indicate the abdominal ventral plates (segments) that are rendered visible through the clear abdominal dorsal cuticle due to depletion of fat body.

gut preparations were made from larvae following dsRNA treatments. RNAi for *TcCHS-B*, which is known to be the sole contributor to PM-associated chitin (Arakane et al., 2005), was included as a positive control for monitoring the loss of chitin. When gut preparations were stained with FITC-CBD to detect chitin in the PM, there was a near complete loss of chitin in the PM in midguts of animals previously injected with dsRNA for either *TcUAP1* or *TcCHS-B*. No loss of chitin staining was apparent in the PM of larvae after RNAi for *TcUAP2* compared to control *dsTcVer*-treated animals (Fig. 7). These results support the hypothesis that the major contributor to the synthesis of UDP-GlcNAc needed for peritrophic matrix chitin synthesis is *TcUAP1* and not *TcUAP2*.

4. Discussion

4.1. A unique UAP gene duplication in the beetle lineage

A bioinformatics search of the *T. castaneum* genome database identified two genes encoding UAPs. In contrast, we could find only one UAP gene in each of the other fully sequenced arthropod genomes examined, including *D. melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Nasonia vitripennis*, *Bombyx mori*, *Apis mellifera*, *Acyrtosiphon pisum*, *Pediculus humanus* and *Daphnia magna*. The two *T. castaneum* UAP genes share ~60% sequence identity at both the nucleotide and encoded protein levels. This degree of amino

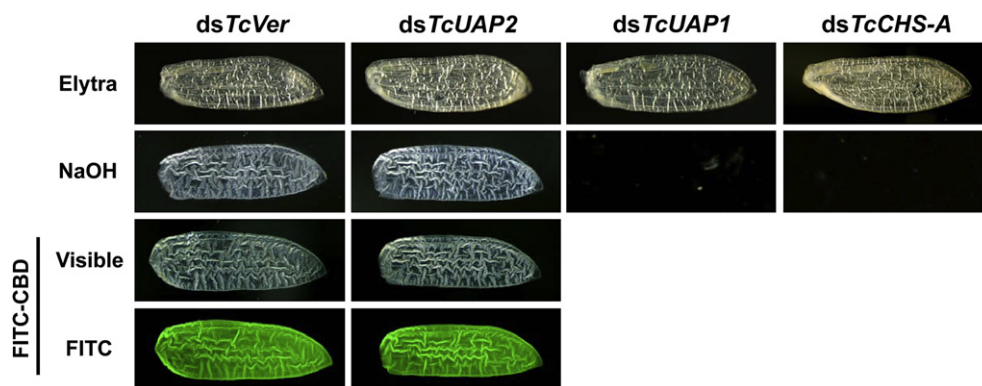


Fig. 6. FITC-CBD staining of elytral cuticle after digestion with NaOH to remove cuticular proteins. One-d-old pupae were treated with dsRNAs for *TcUAP1*, *TcUAP2* or *TcCHS-A* and the elytra were dissected out from 5-d-old pupae. The elytra from *dsTcUAP1*- and *dsTcCHS-A*-treated animals were fragmented and dissolved by the treatment (10 M NaOH at 95 °C for 5 h). In contrast, elytra from *dsTcUAP2*-treated insects retained their original shape even after NaOH treatment, and showed normal staining by FITC-conjugated chitin-binding protein (FITC-CBD) when compared with elytra from *dsTcVer*-treated control insects.

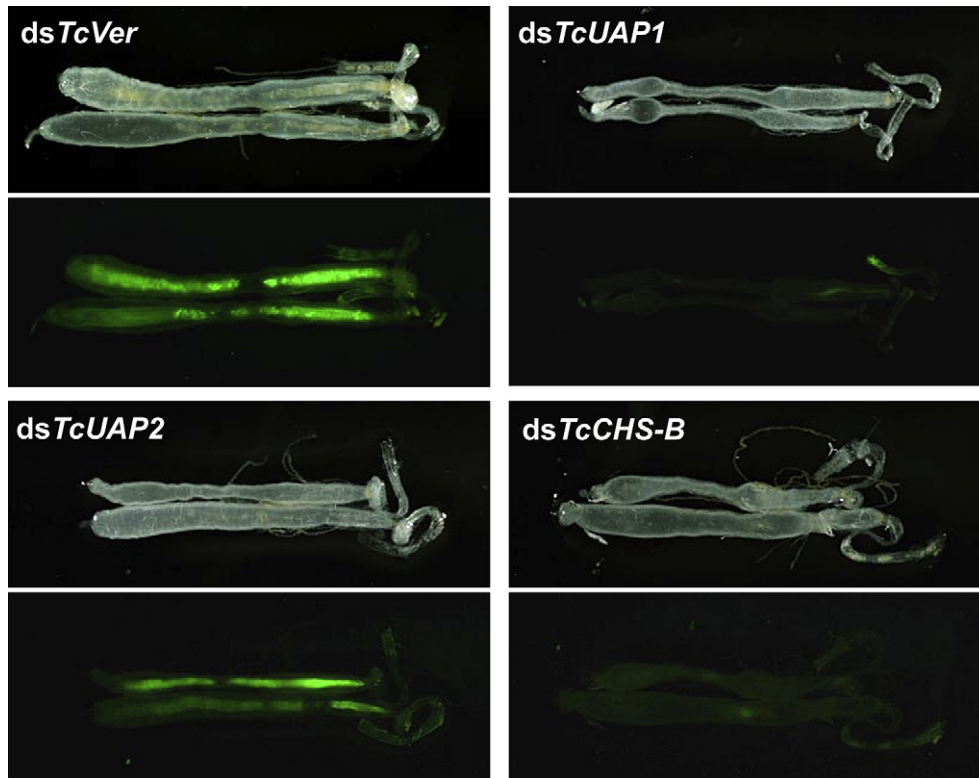


Fig. 7. Effect of injection of dsRNA for *TcUAP1* and *TcUAP2* on chitin staining of the peritrophic matrix with FITC-CBD. Guts were dissected out from feeding larvae three days after injection of dsRNAs for *TcUAP1* or *TcUAP2*. dsRNAs for *TcCHS-B* or *TcVer* were injected to serve as positive and negative controls, respectively. Midguts from *dsTcUAP1* or *dsTcCHS-B*-treated insects exhibited little or no FITC fluorescence, while midguts from *dsTcUAP2*-treated insects showed fluorescence intensities similar to those of *dsTcVer*-treated control insects.

acid sequence conservation is greater than that between either one of the *T. castaneum* UAPs and other insect UAP orthologs, which is in the range of only 47–55%. The absence of a second UAP copy in other species indicates that the two *T. castaneum* paralogs arose by duplication in recent evolutionary time, even though they are not physically linked in the genome. As expected, phylogenetic analysis of UAP proteins from insects of different orders also supports the notion of a recent duplication of this gene in *T. castaneum*. To investigate the possibility that this gene duplication may be unique to coleopterans, we searched the gut EST database of another beetle, *Tenebrio molitor* (B. Oppert, unpublished data), and identified an ortholog of *TcUAP1* but not an ortholog of *TcUAP2*. However, the expression level of *TcUAP1* is much greater than that of *TcUAP2* in larval gut tissue of *T. castaneum* (Fig. 3) (Morris et al., 2009). If this observation also extends to *T. molitor*, then our failure to detect the ortholog of *TcUAP2* in the *T. molitor* EST database may be attributable to a similar lower abundance of these transcripts in the gut of this beetle. At any rate, it is clear that in the group of arthropods analyzed so far, which includes representatives from coleopteran, dipteran, hymenopteran, hemipteran, lepidopteran, tick and crustacean lineages, *T. castaneum* is the sole exception to the “single-copy UAP gene” rule for arthropods.

Like *T. castaneum*, the nematode *Caenorhabditis elegans* has two UAP genes. Being 98–99% identical at the nucleotide level, these must have derived from a very recent gene duplication event. At least one of these nematode genes is required for the osmoregulatory function of the chitinous eggshell (Gönczy et al., 2000), but the dsRNAs used for knockout studies of this gene to date do not differentiate between the two mRNAs, and the unique functions of each gene, if any, have not been resolved. The human genome also contains two paralogs of the UAP gene, UAP1 and UAP1-L, each with

alternative splice variants. Two isoforms of the proteins encoded by the human UAP1 gene are derived from alternatively spliced transcripts, one of which includes a 17-amino acid insertion in a loop structure, denoted as the I-loop in the C-terminal domain (Wang-Gillam et al., 1998; Peneff et al., 2001). UAP gene duplication appears to be prevalent in vertebrate species, including mouse, zebrafish and zebra finch, as well as human, as revealed by sequence similarity searches of the respective genome databases.

4.2. Functional divergence after UAP gene duplication

Both of the human UAP1 isoforms, AGX1 and AGX2, can make both UDP-GlcNAc and UDPGalNAc using GlcNAc-1-P or GalNAc-1-P as substrates (Szumilo et al., 1996; Peneff et al., 2001). The k_{cat}/K_m values of AGX1 and AGX2 were nearly the same, with the value for UDP-GlcNAc as the substrate being about an order of magnitude greater than that for UDPGalNAc as the substrate (Peneff et al., 2001), indicating the versatility of this enzyme in making both products. This may be a general property of UAPs from all species. In fact, *A. aegypti* UAP can utilize the non-acetylated substrate glucose-1-P, even though the K_m for this substrate is 15 times higher than the K_m for the acetylated substrate, GlcNAc-1-P (Kato et al., 2005).

Since many arthropods produce only a single UAP isoform, it is likely that at least some arthropod UAPs are involved in the production of both UDP-GlcNAc and UDP-GalNAc, both of which are needed even in tissues that do not participate in chitin synthesis because of their requirement for glycosylation of protein and other substrates. Since many insects have a single UAP gene, the presence of two genes in *T. castaneum* and humans, encoding two UAP-like proteins differing substantially in their amino acid sequences,

remains an enigma. It is possible that these sequence differences influence the relative affinity of the two UAP isoforms for the two phosphorylated sugar substrates, GlcNAc-1-P and GalNAc-1-P. This could lead to different rates of production of UDP-GlcNAc and/or UDP-GalNAc in different tissues to match their specific needs.

Crystal structures of the two isoforms of human UAP1 (AGX1 and AGX2) in complexes with either of the two alternative substrates, UDP-GlcNAc and UDP-GalNAc, have identified the residues critical for substrate binding, including those that bind to the acetyl group on the 2-amino group and the C4-hydroxyl groups of the hexosamine portion of UDP-GlcNAc/UDPGalNAc substrates (Peneff et al., 2001). All but one of the residues that have been shown to interact with the UDP-GlcNAc/UDPGalNAc substrate in human UAP1 are conserved in TcUAP1 and TcUAP2 (see Fig. 1). One significant difference between the human UAP and TcUAPs is that the alanine at position 329 in human UAP1 is replaced by a cysteine in both TcUAPs (position 330; see Fig. 1). All other arthropod UAP proteins examined, as well as a protein encoded by the second human UAP gene, UAP1-L, have a cysteine in this position. The only other species to have an alanine in this position is *C. elegans* (both CeUAP1 and CeUAP2). It is most interesting to point out that this alanine backbone amide in the human UAP1 is one of two amino acid residues that hydrogen bond with the hydroxyl group on the C4 of glucosamine/galactosamine moiety, the other amino acid being a glycine at position 290 which is conserved in UAPs from all species including humans, insects, *C. elegans* and *Arabidopsis thaliana*. It is not clear whether a cysteine in this position can similarly interact with both sugar substrates or how it influences the affinity of the UAP enzyme for the two substrates. At any rate it is unlikely that the two *T. castaneum* enzymes will differ in their substrate preferences because all the critical amino acids known to interact with the two substrates/products are the same in both TcUAP1 and TcUAP2.

Both TcUAP enzymes can be modeled on the human AGX1 template to yield proteins with the same overall architecture (Peneff et al., 2001; our unpublished observations). The major difference is in the N-terminal domain, where TcUAP has only two α -helices, whereas TcUAP2 has four. However, these differences do not seem to affect the architecture of the central catalytic domain. Since the two TcUAP genes are on different chromosomes and under the control of different promoters, additional controls may exist that regulate their expression in different tissues and/or developmental stages.

The UAP gene of *D. melanogaster* has two promoters and gives rise to two splice variants, with sequences in the first intron of the UAP gene serving as an alternative promoter for the transcript that gives rise to the shorter isoform (<http://flybase.org/cgi-bin/gbrowse/dmel/?Search=1;name=FBgn0259749>). This results in the production of two proteins, one with an additional 37 amino acids at the N-terminus. However, this additional stretch is unlikely to change the substrate preference of the two *Drosophila* UAP enzymes because the catalytic domain is in the middle of the protein and includes all the residues involved in binding of both substrates.

The finding that there are two UAP genes in *T. castaneum* was initially reminiscent of the presence of two CHS genes in insects, which have distinct, tissue-specific expression and physiological functions (Kato et al., 2006; Arakane et al., 2005, 2008; Hogenkamp et al., 2005; Zimoch et al., 2005; Chen et al., 2007), but the work reported herein disproves any such correlation. The TcCHS-A and TcCHS-B genes are expressed mutually exclusively in the epidermal versus midgut epithelial tissues, respectively, and the corresponding proteins CHS-A and CHS-B have specialized functions in synthesizing chitin in the cuticle and the PM, respectively. In contrast, TcUAP1 appears to be the major, if not

exclusive, contributor to chitin synthesis in both the epidermis and midgut. The TcUAP1 gene is expressed in cuticle-forming tissues such as the integument, hindwing and elytron, as well as in the midgut (Fig. 3B, D). TcUAP1-specific RNAi is followed by loss of chitin staining in the PM, and by loss of chitin-dependent resistance to dissolution of elytra by NaOH. No such effects were observed after RNAi specific for TcUAP2, with elytra and PM both showing normal chitin staining, even though these two tissues normally contain TcUAP2 transcripts (Fig. 3D). Insects died at the time of molt after TcUAP1 RNAi, presumably because chitin synthesis is required to form a new cuticle with sufficient mechanical strength to break through the old cuticle. The phenotypes are very similar to those that we have previously reported for animals subjected to RNAi for TcCHS-A, which catalyzes cuticular chitin synthesis (Arakane et al., 2005).

4.3. TcUAP2 has evolved an essential function that is not compensated by TcUAP1

Although TcUAP2 does not appear to be required for chitin synthesis in cuticular tissues such as the elytron or in the midgut, RNAi studies suggest an essential role for TcUAP2 in the larval and adult stages. TcUAP2 transcripts as well as those of TcUAP1 are detectable in several larval and adult tissues, including midgut, hindgut, testes and ovaries (Fig. 3). We cannot discount the possibility of a minor, albeit non-essential, contribution to the synthesis of chitin in the cuticle or PM by TcUAP2, but the timing of developmental arrest and the morphology of the moribund animals after TcUAP2 RNAi are not consistent with specific effects on cuticle turnover or molting, and are distinctly different from those of insects subjected to RNAi for TcUAP1. We suspect that the lethal effects following RNAi for TcUAP2 are due to defective glycosylation of proteins or secondary metabolites, whose functions are critical for insect survival, rather than to any minor contribution it might make to chitin synthesis, as we have seen no significant changes in chitin staining either in the elytra or the PM following RNAi for this gene.

Since only TcUAP1 is required for chitin synthesis in both the cuticle and gut tissues, this must be the more ancient UAP gene ortholog in other insects. The TcUAP2 paralog probably acquired a new function or took over some of the non-chitin-related functions of the progenitor gene after the duplication event. For example, TcUAP2 may be the major provider of UDP-GalNAc in all tissues in *T. castaneum*, whereas in other arthropods a single enzyme might catalyze the production of both UDP-GlcNAc and UDP-GalNAc.

The effects of knockdown of either UAP transcript in mature adult beetles differ from those of TcCHS-A (the latter having no vital role in mature adults) but are at least superficially similar to those of TcCHS-B. In the case of TcUAP1, the similarity probably reflects their shared function. Both TcUAP1 and TcCHS-B are required for PM chitin synthesis and PM integrity, and are therefore essential for digestion and nutrition in the adults, as well as in larvae. In these cases, fat body autophagy and death probably stem from cessation of normal digestion and absorption of nutrients. The finding that RNAi for TcUAP2 yields a very similar phenotype (fat body depletion and death) is not easily explained by postulating that a PM-related disruption of digestion and nutrient absorption occurs, since TcUAP2 is not needed for production of PM chitin, at least in larvae (Fig. 7). The superficial similarity in the adult lethal syndromes of TcUAP2 and TcUAP1 (or TcUAP2 and TcCHS-B) could indicate that TcUAP2 has some important non-chitin-related function whose disruption leads to loss of appetite, or inability to feed or to digest, absorb or utilize dietary nutrients. This would, in turn, result in starvation, loss of fat body and death.

Using whole-transcriptome microarray analysis, Morris et al. (2009) found that *TcUAP1* but not *TcUAP2* is highly expressed in the *T. castaneum* larval midgut, a finding that is consistent with our observations and inferences. Our RNAi results clearly show that the essential functions of TcUAP2 are not fulfilled by TcUAP1, either because of differences in tissue-specificity or functional specialization of these two proteins. These multiple functions are apparently met by protein(s) encoded by a single UAP gene in other arthropod species. For example, Araujo et al. (2005) and Schimmelpfeng et al. (2006) observed CNS defects and eye developmental abnormalities in *D. melanogaster* UAP mutants in addition to cuticular defects. We suspect that an essential glycoprotein(s), GPI-anchored protein or glycoconjugate is involved in the TcUAP2 pathway. The depletion of the fat body after RNAi for *TcUAP1* or *TcUAP2* in adults suggests that different physiological functions related to fat body formation may be affected in these studies. RNAi for either UAP gene could affect multiple glycosylated proteins or metabolites including those that are components of the PM or hindgut. For example, several of the *T. castaneum* PM proteins have potential glycosylation sites and/or mucin domains, which are likely to be glycosylated (Jasrapuria et al., 2010). Failure of glycosylation of these proteins could result in an altered PM or affect a hindgut function, leading to defects in digestion or absorption of nutrients unrelated to any effect on chitin metabolism. Whether these are the targets affected by loss of TcUAP1/TcUAP2 remains to be investigated. Any protein or secondary metabolite that needs to receive a GlcNAc or GalNAc residue from UDP-GlcNAc or UDP-GalNAc could potentially be affected by depletion of TcUAP1 and/or TcUAP2. Post-translational modifications of protein involving the addition of a single O-GlcNAc have been shown to regulate cellular responses to insulin, activate specific genes and influence cellular responses to changes in the nutritional state of the organism (Slawson et al., 2006). As indicated earlier, the two TcUAP enzymes may have different substrate specificities that may be crucial for providing substrates for glycosylation of specific proteins or other substrates in specific tissues or intracellular locations. Future studies may shed light on the precise mechanisms underlying the loss of fat body following RNAi of the two *TcUAP* genes.

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Supplementary table

Supplementary table associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2010.09.011.

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