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Developmental expression and phylogenetic conservation of alternatively spliced forms of the C-terminal Binding Protein corepressor.

Priya Mani-Telang[1,3] and **David N. Arnosti**[1,3,5]

1 *Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, Michigan 48824*

3 *Program in Cell and Molecular Biology, Michigan State University, East Lansing, Michigan 48824*

Abstract

The C-terminal binding protein (CtBP) is an evolutionarily conserved transcriptional corepressor found in multicellular eukaryotes. Multiple forms of the protein are typically found in animal cells, produced from separate genes and by alternative splicing. CtBP isoforms have also been implicated in cytoplasmic functions, including Golgi fission and vesicular trafficking. All forms of CtBP contain a conserved core domain that is homologous to α-hydroxyacid dehydrogenases, and a subset of isoforms ($CtBP_L$) contain extensions at the C-terminus. Despite distinct developmental profiles and knockout phenotypes in the mouse, the properties of different isoforms of the protein are found to be similar in many transcriptional assays. We have investigated the expression and conservation of distinct isoforms of the CtBP protein in insects, and find that the expression of multiple, developmentally regulated isoforms is widely conserved. In a variety of *Drosophila* species, the relative abundance of $CtBP_L$ to $CtBP_S$ drops sharply after embryogenesis, revealing a conserved developmental shift. Despite the overall lower levels of this isoform, bioinformatic analysis reveals that exons encoding the C-terminal extension in $CtBP_L$ are conserved from Diptera to Coleoptera, suggesting that the $CtBP_L$ isoform contributes an important, evolutionarily conserved function.

Keywords

C-terminal binding protein; CtBP; transcription; corepressor

Introduction

The C-terminal binding protein (CtBP) is an evolutionarily conserved factor that has been implicated in a variety of cellular processes, including transcriptional repression, Golgi function, and vertebrate retinal synapse activity (Chinnadurai, 2005). Originally identified by its ability to interact with the C-terminus of the adenovirus E1A protein, CtBP has been shown to directly bind to a variety of transcription factors in vertebrate cells and in *Drosophila*, and recruit chromatin-modifying factors including histone deacetylases and histone demethylases (reviewed in Turner and Crossley, 2001;Chinnadurai, 2003). CtBP proteins share a high degree of similarity within a central domain comprised of an NAD-binding domain and a substrate-binding fold. The proteins form dimers, and demonstrate extensive structural similarity to NAD-dependent dehydrogenases (Kumar et al., 2002;Nardini et al., 2003). CtBP proteins also

[5]Corresponding author: Email: arnosti@msu.edu FAX 517-353-9334

possess C-terminal sequences of variable lengths that are likely to be unstructured (Nardini et al., 2006).

CtBP proteins exhibit a weak NAD-dependent catalytic activity in vitro, however the physiological relevance of this activity is unknown (Kumar et al., 2002;Balasubramanian et al., 2003;Barnes et al., 2003). NAD binding has also been suggested to regulate CtBP allostery, permitting the interaction of the protein with binding partners. In vitro, association of CtBP with E1A proteins is stimulated by NAD and NADH, suggesting a possible molecular switch that might regulate CtBP activity (Kumar et al., 1987;Barnes et al., 2003). Differential affinity of the protein for NADH relative to NAD has been suggested to endow CtBP with the potential to respond to differing intracellular levels of these cofactors, possibly linking gene regulation to cellular redox states (Zhang et al., 2002). A possible lysophosphatidic acid acyl transferase activity relevant to membrane trafficking has also been ascribed to one form of CtBP (CtBP3/CtBP1-S/BARS), however this result has been disputed (Weigert et al., 1999;Gallop et al., 2005).

Distinct CtBP isoforms are expressed as a result of alternative splicing, alternative promoter usage, and different genes. In vertebrates, the *ctbp1* and *ctbp2* genes are expressed in overlapping patterns during development and exhibit distinct functions. *ctbp1* knockout mice are viable, but are smaller and show increased postnatal mortality, while the *ctbp2* mutation is embryonic lethal (Hildebrand and Soriano, 2002;Van Hateren et al., 2006). The rat CtBP1 isoform termed CtBP1-S/CtBP3/BARS lacks a short region at the N terminus; this protein has been implicated in membrane fission events that are required for Golgi trafficking and Golgi fragmentation during mitosis (reviewed in Corda et al., 2006). In vertebrates, the RIBEYE splice form of CtBP2 produces a protein containing CtBP residues fused to a unique N terminus; this protein is localized to synaptic vesicles of the retina (Schmitz et al., 2000).

Posttranslational modifications and association with other binding proteins have been shown to regulate the stability, activity and localization of CtBP proteins in vertebrates. Some of these modifications target the central conserved region of the protein; the Pak1 kinase phosphorylates CtBP1 at Ser158, stimulating nuclear to cytoplasmic translocation and downregulating CtBP repression activity (Barnes et al., 2003). Other modifications are targeted to the C-terminal nonconserved portion of the protein; phosphorylation of CtBP1 ser422 by the HIPK2 kinase promotes degradation of the protein, whereas SUMOylation of the C-terminus is required for nuclear localization of CtBP1 ()(Kagey et al., 2003;Lin et al., 2003;Zhang et al., 2005). In addition to being covalently modified, the C-terminus can also serve as the binding target for a PDZ-domain containing protein, neuronal nitric oxide synthase, that drives cytoplasmic localization of the CtBP1 (Riefler and Firestein, 2001).

In contrast to vertebrates, distinct *Drosophila* CtBP proteins are produced from a single gene. Two major isoforms, termed $CtBP_L$ and $CtBP_S$, differ by the presence or absence of a ~90 amino acid extension at the C-terminus, which, although of similar size and amino acid composition, is not homologous to C-terminal extensions found in vertebrate CtBP proteins (Poortinga et al., 1998;Nibu et al., 1998a). In light of the fact that the unstructured C-terminus can play a regulatory role in vertebrates, it seems possible that *Drosophila* $CtBP_L$ may be subject to similar covalent modifications as those found in vertebrates, but currently there is little understanding of the biological importance of the different isoforms. In vitro, both $CtBP_L$ and $CtBP_S$ are able to bind to short-range transcriptional repressors such as Knirps and Krüppel, and in transcriptional assays, both isoforms exhibit similar activities (Sutrias-Grau and Arnosti, 2004;Fang et al., 2006). Therefore, we have utilized biochemical and phylogenetic analysis to study expression of the protein in disparate orders to gain more insight into the significance of distinct isoforms of this widely conserved protein. Biochemical and

phylogenetic evidence indicates that the alternatively spliced $CtBP_L$ isoform represents a conserved, developmentally regulated form of the protein, suggesting a specific functional role for this protein.

Materials and Methods

Insect stocks and lysate preparation

The fly stocks used in this study were: *D. melanogaster* yw^{67} (Arnosti lab), *D. sechellia*, *D. mojavensis* (Tucson *Drosophila* Stock Center), *D. virilis* (Dr. Scott Pitnick). *Tribolium castaneum* was a gift from Dr. Susan Brown, *Anopheles gambiae* from Dr. Ned Walker and *Apis mellifera* from Dr. Zachary Huang. All flies were maintained on standard cornmeal/molasses food and embryos collected at 25°C on apple juice/agar. For developmental expression analysis, staged embryos were collected, dechorionated and resuspended in lysis buffer (150mM NaCl, 50mM Hepes, pH 7.9, 10% glycerol, 0.1mM EDTA with Complete mini-EDTA free protease inhibitor cocktail tablet, Roche) and sonicated using a Branson-250 sonifier. Larvae, pupae and adults were homogenized in lysis buffer with a steel pestle and then sonicated under the same conditions. Lysates were cleared by centrifugation and total protein concentration of the supernatant was measured by a Bradford assay with BSA as the standard. The supernatant was mixed with Laemmli sample buffer for SDS-PAGE analysis. For identification of CtBP isoforms in bee, beetle, and mosquito and flies, whole adult animals were homogenized in Laemmli sample buffer for SDS-PAGE analysis.

Western Blot Analysis

Immunoblotting was performed using 10% SDS-PAGE gels in a tank transfer system (Bio-Rad Mini Trans-Blot® Cell) and proteins were transferred to Immuno-Blot™ PVDF membrane (Bio-Rad). Antibody incubation was performed in TBST (20mM Tris-HCl, pH 7.5, 120mM NaCl, 0.1% Tween-20) supplemented with 5% non fat dry milk as a blocking agent. Rabbit polyclonal antibodies used to detect CtBP (1:10,000) and monoclonal mouse antibody for tubulin (1:6000, Iowa Hybridoma Bank) were visualized using HRP-conjugated secondary antibodies (Pierce) and SuperSignal® West Pico chemiluminiscent substrate (Pierce). Western blots shown are representative of at least three biological replicates for each experiment.

Antibodies and recombinant CtBP proteins

Polyclonal anti-CtBP antibodies were generated as described in Struffi et al. (2005). For the production of recombinant proteins, the cDNAs for $CtBP_L$ and $CtBP_s$ bearing two Flag epitope tags at the C-terminal end was cloned into the pET15b expression vector and used to transform *E.coli* BL-21 cells. Expression of bacterial proteins was induced by treating log-phase cultures with 0.4mM IPTG. The cells were then sonicated in lysis buffer, centrifuged and supernatant was dissolved in Laemmli sample buffer for western analysis.

RT-PCR measurements of splice form abundances in embryos and adults

Total RNA of *D. melanogaster* embryos (stage 0–12) and adults was isolated by tissue homogenization in Trizol reagent (Sigma) according to the manufacturer's protocol. RNA was treated with Rnase-free DNase (RQ1, Promega) to remove contaminating genomic DNA. RT-PCR was performed using AccessQuick™ RT-PCR System from Promega. Transcripts for $CtBP_L$ were amplified using primer pairs DA1147 – 5’ CCCCACAGTACAACCAACCT 3’ and DA1148 – 5’ TCCGTTTTTATGCTCGATGA 3’, CtBPs using primer pairs DA 1146 – 5’ CTCAACGAGCACAACCATCATTTAATC 3’ and DA 1150 – 5’ CTCTACTTTTCTTGATTTGATATCATTTGTAG 3’ and total CtBP was amplified using primer pairs DA 1146 - 5’ CTCAACGAGCACAACCATCATTTAATC 3’ and DA 1151 –

5'GCACGTCTGGAATATTGCCGAC 3'. All primer pairs spanned an intron such that amplification of contaminating genomic DNA could be distinguished from the RT-PCR amplified products. The RT step was performed at 45°C for 45 mins followed by 30 cycles of PCR in a 25 μl reaction mix for 94°C for 1min, 56°C for 1 min and 72°C for 1 min. PCR products were visualized by agarose gel electrophoresis and quantitated using BioRad Quantity One software Version 4.4.1. The data shown in Figure 1C is a representative result of RT-PCR analysis of biological triplicates that were each analyzed at least two times.

Sequence alignments

To determine the conservation of CtBP exons in diverse insect genomes we searched the Flybase database (Release 4.2) using FLYBASE BLAST for the assembled genomes of *Drosophila melanogaster*, *D. sechellia*, *D. persimilis*, *D. mojavensis*, *D. virilis*, *D. grimshawi*, *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera* and *Tribolium castaneum.* Matches to conserved exons 1–4 of CtBP were obtained for *D. sechellia (AAKO01000254.1) D. persimilis* (AAIZ01000471), *D. mojavensis* (contig_8705), *D. virilis* (contig_15233), *D. grimshawi* (contig_21987), *A.gambiae* (AAAB01008805), *Aedes aegypti* (supercontig_1.155), *A.mellifera* (AADG05006060) and *T. castaneum* (CM000284.1). These automated alignments generally did not identify exons 6 and 7, however, therefore sequences 3' to the conserved exons were searched in all three reading frames for conserved coding information and aligned using Clustal W. Predicted gene sequences for *A. mellifera* (XM_392682) and *T. castaneum* (XP_972241) were included in these alignments.

Results

Expression of CtBP isoforms in *Drosophila*

Four major *CtBP* transcripts are detected ubiquitously during development and are predicted to produce proteins of 383, 386, 476 and 479 amino acids (Poortinga et al., 1998;Nibu et al., 1998b;Sutrias-Grau and Arnosti, 2004). To analyze endogenous CtBP proteins in *Drosophila*, we generated polyclonal rabbit antibodies against $CtBP_L$ protein (aa1-479) expressed in *E.coli*. The antibodies are specific and detect proteins of the expected sizes in embryonic extracts, approximately 42 ($CtBP_S$) and 50 KDa ($CtBP_L$) (Fig. 1A). Immunostaining revealed that CtBP proteins are ubiquitously present in the nuclei of pre- and post-blastoderm embryos and imaginal discs from third instar larvae (data not shown).

To analyze the developmental expression profile of CtBP isoforms, we analyzed soluble extracts from different developmental stages of the fly (Fig. 1B). Both $CtBP_L$ and $CtBP_S$ isoforms are detected throughout the first 15 hours of embryogenesis, with relatively higher level of $CtBP_S$ than $CtBP_L$ (antibody recognition of $CtBP_L$ is expected to be equal or better than that of $CtBP_S$ because the two proteins are virtually identical in the central domain, and the antibody was raised against $CtBP_L$). The relative levels of $CtBP_L$ to $CtBP_S$ drop further after embryogenesis, showing weak expression of $CtBP_L$ in the larva, pupa, and adult (Fig. 1B). The lower abundance of $CtBP_L$ in postembryonic stages is not simply due to sequestration of the protein in an insoluble form, because similar low levels of $CtBP_L$ were observed in whole animal extracts prepared in boiling SDS (discussed below).

We measured the relative levels of specific CtBP mRNA splice forms in embryonic and adult stages to determine if this developmental switch reflects a change in alternative mRNA isoform abundance. Primer pairs specific to the $CtBP_S$, $CtBP_L$, and to a region of the gene common to both isoforms were used in RT-PCR reactions. The absolute amounts of $CtBP_S$ and $CtBP_L$ RT-PCR products are not directly comparable because different primer sets were used, however the relative ratios in different stages of development are informative. The ratio of $CtBP_S$ to

$CtBP_L$ transcripts undergo a marked shift between these two stages, with relative levels of $CtBP_S$ increasing approximately 4 fold with respect to $CtBP_L$ (Fig. 1C). This change suggests that the developmental protein profile may be largely determined by changes in the abundance of distinct splice forms of the mRNA. Additional post-transcriptional effects may also contribute to the decreased $CtBP_L$ protein levels observed.

Identification of conserved $CtBP_L$-specific coding information

We examined genomic sequences of 10 different insects representing >300 million years of evolutionary divergence – the fruit flies *D. melanogaster*, *D. sechellia*, *D. persimilis*, *D.mojavensis*, *D. grimshawi* and *D. virilis*, the mosquitoes *Anopheles gambiae and Aedes aegypti* (Diptera), the honey bee *Apis mellifera* (Hymenoptera) and the red flour beetle *Tribolium castaneum* (Coleoptera) - to determine if these organisms might also express diverse isoforms of CtBP. Analysis of putative open reading frames 3' of core conserved CtBP sequences identified regions homologous to *D. melanogaster* exons 6 and 7, which encode the C-terminal extension of $CtBP_L$ (Fig. 2A). In *Drosophila* species, the sequences of exon 6 appear to be separated from an upstream exon by a ~3 kbp intron, while the intron is of smaller size in mosquito and beetle. In the honey bee, this intron appears to have been entirely eliminated. The overall similarity among putative C-terminal coding regions is clearly lower than that observed for the core CtBP sequences, suggesting a lowered level of constraint. However, the similarities include several distinctive motifs involving less abundant amino acids, not simply tracts of repeating residues that would show similarities by chance. Splice signals following the terminal codons for exon 5 (YPEG), are conserved in all *Drosophila*, as well as lower Diptera and *Tribolium*, suggesting that the downstream coding information is likely to be incorporated into mRNAs (Fig. 2B). Splice acceptor sites are present immediately 5' of the conserved LNGGYYT coding region of exon 6 in *Drosophila* species. A conserved splice acceptor sequence is not found directly 5' of I/VNGGY coding sequences present in *Tribolium* and *Anopheles*, raising the possibility that acceptor sites in alternative locations may be used (Fig 2B). In the bee, the information for the tail extension seems to be fused to the core sequences, supporting the notion that these are indeed coding sequences. Similar to the case with vertebrate CtBP proteins, the predicted C-terminal extensions of these CtBP isoforms are probably unstructured in solution. The sequences are rich in disorder promoting amino acids (ala, gly, pro, ser) and are predicted to not assume a globular structure by the GlobPlot program (not shown) (Linding et al., 2003).

Developmental expression of alternative isoforms in *D. mojavensis* and *D. virilis*

The presence of the regions correlating to *D. melanogaster* exons 6 and 7 does not in itself reveal whether distinct CtBP isoforms are produced, therefore we measured CtBP protein levels in embryos, larvae, pupae and adults from *D. mojavensis* and *D. virilis*, which are estimated to have shared the last common ancestor with *D. melanogaster* about 40–60 million years ago. Western blot analysis revealed that two major bands of sizes similar to $CtBP_S$ and $CtBP_L$ were present in these species (Fig. 3). The relative abundance of the $CtBP_L$ isoform decreases in larval and pupal stages, staying low in *D. mojavensis* in the adult, but increasing again in adult *D. virilis*. While differing in details, these changes suggest that developmental changes in relative abundances of CtBP isoforms are a conserved feature in Drosophilids.

Expression of CtBP isoforms in diverse orders

To determine whether expression of $CtBP_S$ and $CtBP_L$-like isoforms is generally conserved in insects, we measured expression of CtBP proteins in organisms whose sequenced genomes had been examined for $CtBP_L$-specific coding information (Fig. 2A). Crude extracts from adults were analyzed by Western blotting, including three *Drosophila* species of increasing

phylogenetic distance from *D. melanogaster*, *Anopheles gambiae* (lower Dipteran), *Apis mellifera* (Hymenoptera), and *Tribolium castaneum* (Coleoptera) (Fig. 4). Relative to *D. melanogaster*, the closely related *D. sechellia*, (diverged ~3 Mya) had $CtBP_S$ and $CtBP_L$ isoforms of the same size. The extracts from the more distantly related species *D. mojavensis* and *D. virilis* (diverged 40–60 ~Mya) contained proteins of similar size to $CtBP_S$ (~42 kDa) and an additional, lower mobility form (60 kDa) that migrated slower than *D. melanogaster* $CtBP_L$. Two proteins were also evident in the mosquito, both of somewhat faster mobility than the *Drosophila* counterparts. Three cross-reacting species were found in the honeybee, all of similar abundance, including one protein of ~25 kDa that migrates considerably faster than $CtBP_S$, similar to a minor species noted in *D. mojavensis* extracts. Only one major isoform of ~50 kDa was detected in extracts from *Tribolium*, similar in mobility to *Drosophila* $CtBP_L$, although upon overexposure, weak bands of faster mobility could be seen. In this figure, the relative levels of $CtBP_L$ and $CtBP_S$ in *Drosophila* appear to be similar, but this is only because the gel was exposed for a long time to bring out the weaker *A. mellifera* bands. A Western blot of the *Drosophila* extracts in which the exposure was shorter reveals that the ratio of $CtBP_L$ to $CtBP_S$ in adults was low in all *Drosophila* species except *D. virilis* (Fig. 4, lanes 8–11), which is consistent with our developmental profiles for *D. virilis* and *D. mojavensis*.

Discussion

In the mouse, the *CtBP1* and *CtBP2* genes have been found to provide overlapping but functionally distinct activities in development (Hildebrand and Soriano, 2002). These different activities might be transcriptionally based, a situation in which homologous genes encode functionally interchangeable products, but the distinct timing and levels of transcriptional activity of the promoters are unique, as has been described for the *Drosophila prd*, *gsb*, and *gsbn* genes (Li & Noll 1994). However, this model cannot be applied to cover all vertebrate CtBP proteins, because the RIBEYE spliceform of CtBP2 and CtBP1-S/BARS splice variant of CtBP1 encode distinct polypeptides, and appear to have acquired unique roles in retinal function and membrane trafficking, respectively (Corda et al., 2006). With respect to the transcriptional regulatory forms of CtBP1 and 2, biochemical studies have identified molecular modifications that may distinguish the two isoforms functionally. CtBP1 is phosphorylated at serine158, a modification that induces nuclear to cytoplasmic translocation (Barnes et al., 2003). CtBP2 has a unique N-terminus that is acetylated, which facilitates nuclear retention of the protein (Zhao et al., 2006). Whether these differences play a role directly in transcription is unclear; both proteins may function similarly when recruited to promoters.

In *Drosophila*, less is known about distinctions among isoforms. Previous work from our and other laboratories has indicated that multiple CtBP isoforms are expressed in *Drosophila*, but no functional distinctions have been drawn between $CtBP_S$ and $CtBP_L$ isoforms until now. Our study provides evidence that the presence of these isoforms is not simply “noise”, for example, aberrant splicing that is tolerated by the system. The evolutionary conservation of multiple isoforms and developmental regulation strongly points to functional differentiation between these proteins. It is striking that all the organisms surveyed express proteins whose size corresponds to the *D. melanogaster* $CtBP_L$ isoform. In addition, all contain conserved coding sequences in their genomes for the unstructured C-terminal extension of the protein, which in the case of mammals is the subject of sumoylation, phosphorylation, and binding of regulatory proteins in mammals. Putative sumoylation signals are conserved in Dipteran sequences (Fig. 2A), suggesting that insect CtBP proteins may similarly be modified by SUMO. All vertebrate CtBP proteins possess some form of C-terminal extension, however the presence of $CtBP_S$ isoforms in insects may indicate that potential regulation by modification of the C-terminus

may not be required, at least in some stages or roles. Additional biochemical and genetic studies will be required to identify possible functional distinctions between these isoforms.

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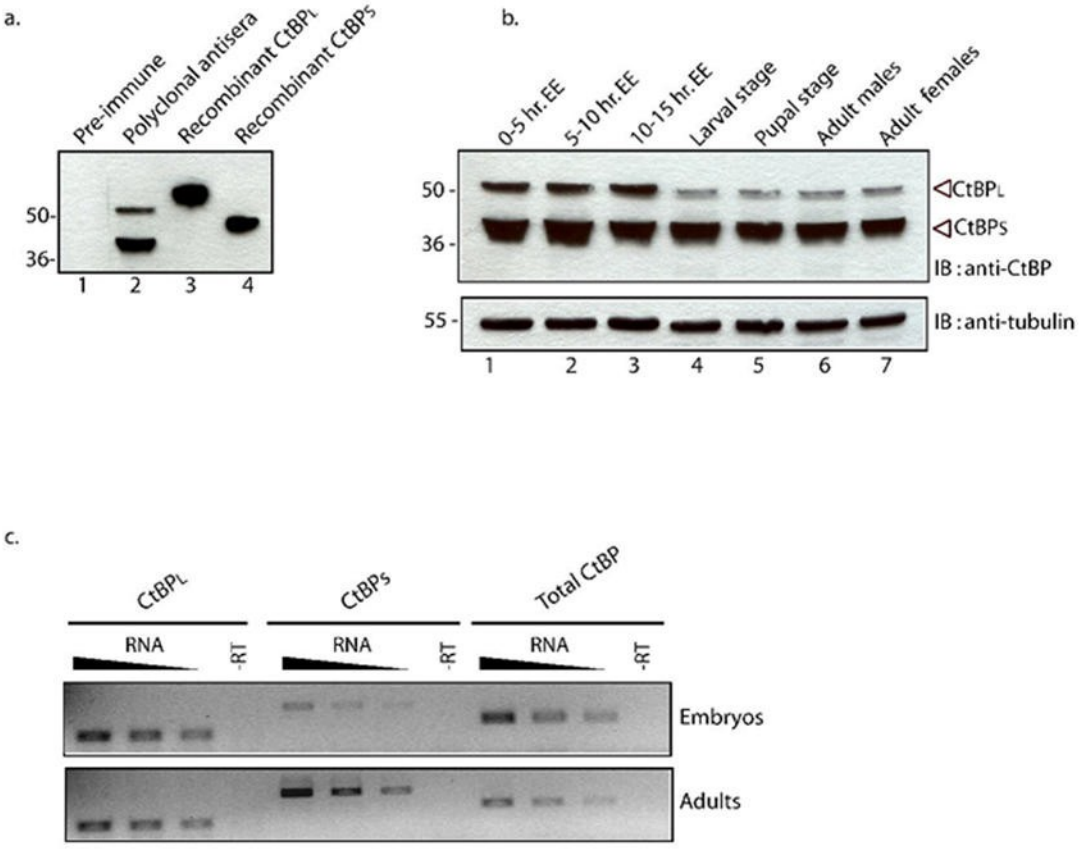

Fig 1. Developmental expression profile of CtBP isoforms in *Drosophila melanogaster*

A.Specificity of α-CtBP antibody tested in Western blot with *Drosophila melanogaster* embryonic extract (lanes 1,2) or bacterial extracts containing recombinant $CtBP_L$ (lane 3) or $CtBP_S$ (lane 4). Preimmune serum did not cross react with any proteins in embryo extract, while α-CtBP recognized two isoforms of approximately 42 and 50 kDa in embryonic extracts. Recombinant proteins migrate slower than endogenous counterparts due the presence of an N-terminal hexahistidine tag and a C-terminal Flag tag. Markers (kDa) are indicated to the left. **B.** Expression of CtBP isoforms in embryos, larvae, pupae, and adults. 50 μg of total soluble protein was loaded on 10% SDS-PAGE and analyzed by immunoblotting with anti-CtBP. Relative $CtBP_L$ and $CtBP_S$ levels were unchanged during embryogenesis. A marked reduction in the relative level of $CtBP_L$ was observed from the larval through adult stages. CtBPs levels remained relatively unchanged throughout the developmental time course. The bottom panel shows β-tubulin as a loading control. **C.** Steady-state levels of CtBP mRNAs measured by RT-PCR analysis. Total mRNA from embryos and adults was reverse transcribed and PCR amplified using primers specific to $CtBP_L$ exons, $CtBP_S$ regions, or a region common to both isoforms as indicated. Reverse transcription reactions were primed with 60, 30, or 15 ng of total RNA, as indicated by triangular symbol. The –RT control reactions were primed with 60 ng of RNA. Based on quantitation of biological replicates, the ratio of $CtBP_L$ to $CtBP_S$ products was measured to be approximately 1:1 in adults compared to 4:1 in embryos.

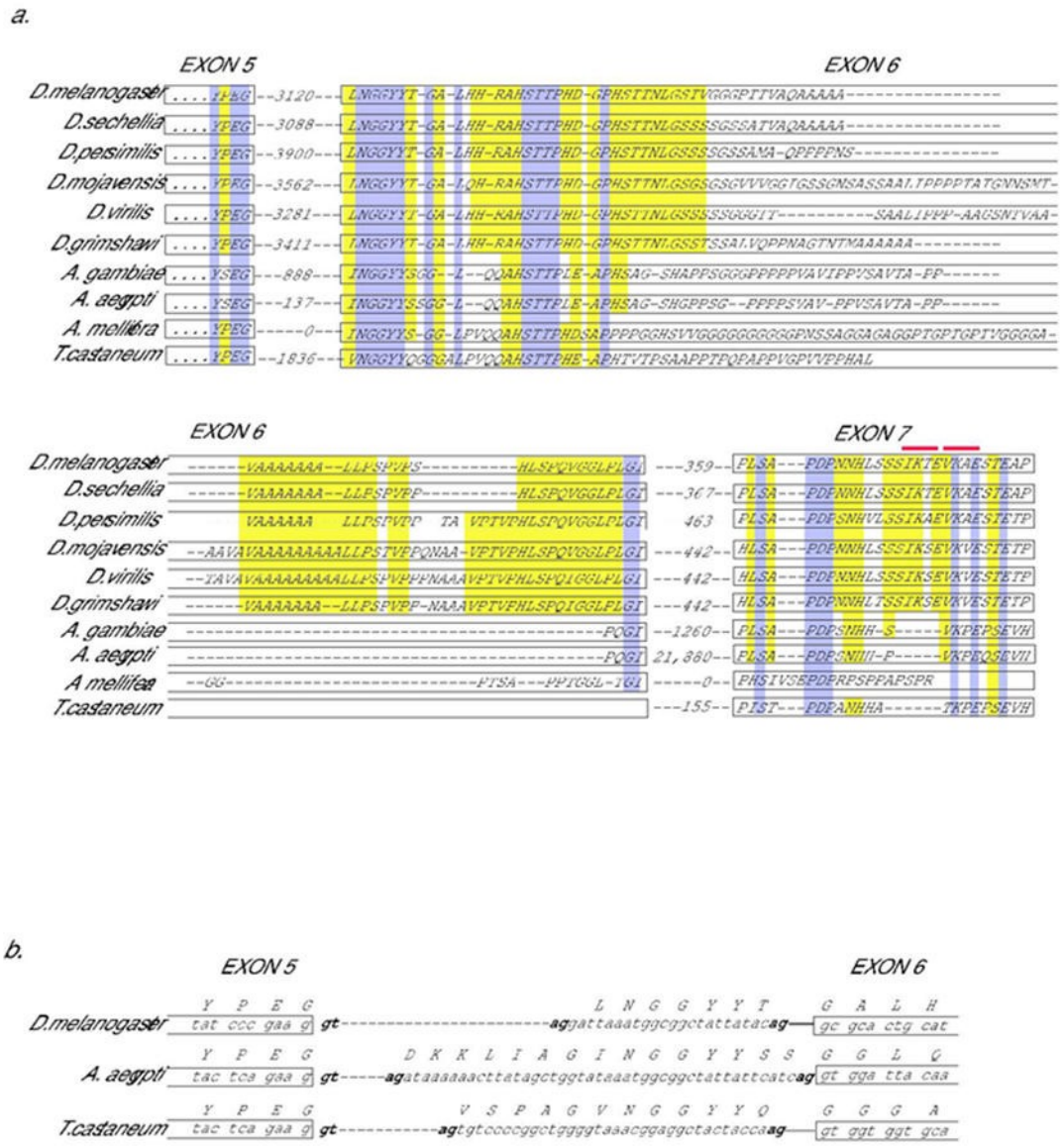

Fig 2. Conservation of coding information for $CtBP_L$-specific C-terminus

A. Peptide coding information present in dipterans, bee, and beetle genomic sequences homologous to alternatively spliced exon 6 and 7 in *Drosophila melanogaster* encoding CtBP "tail" region. Conceptual translations of genomic sequences are shown below sequence of $CtBP_L$, in which YPEG represents the end of the exon 5 coding sequence for the $CtBP_L$ isoform. Predicted intron size in nucleotides is indicated between exons. The introns in *Apis mellifera* have apparently been eliminated. Dark gray (purple) shading indicates widely conserved sequences; light gray (yellow) shading partially conserved sequences. Possible sumoylation sites (I/V K X E) are indicated by gray (red) bars above exon 7 residues. An alternative splice acceptor site 5' of the junction shown for exon 7 would produce an mRNA encoding an additional VSSQS motif at the beginning of exon 7; this sequence is not conserved outside of *Drosophila*, unlike the case shown in 2B. **B.** The cDNA sequences reported for *D. melanogaster* $CtBP_L$ contain alternative splice acceptor sites for the 5' end of exon 6; a sequence isolated from adult head uses a downstream acceptor site (NP_001014617), while a different sequence isolated from embryo uses a more upstream acceptor ((Sutrias-Grau and

Arnosti, 2004)) incorporating the residues LNGGYYT. This portion of the protein is evolutionarily conserved and contains appropriate splice acceptor sequences both 5’ and 3’ of region, thus alternative splicing may be a conserved feature here as well.

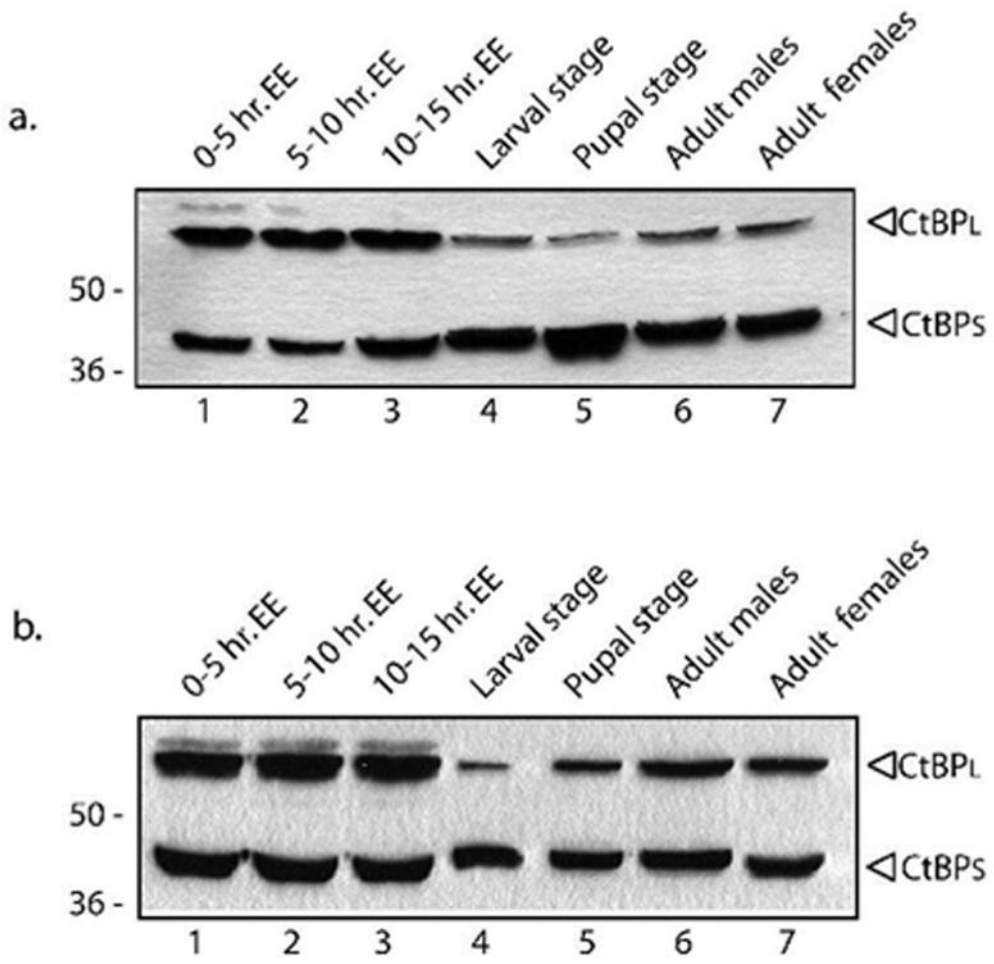

Fig 3. Conserved developmental regulation of CtBP protein expression in *D. mojavensis* and *D. virilis*

Expression of CtBP isoforms in embryos, larvae, pupae, and adults of *D. mojavensis* (A.) and *D. virilis* (B.). As in *D. melanogaster*, two predominant species were observed in both species, but the $CtBP_L$ isoform has a lower mobility (~60kDa vs. 50kDa in *D. melanogaster*). The relative levels of $CtBP_L$ to $CtBP_S$ in the embryo was greater in these species than in *D. melanogaster*, but just as in that species there is a pronounced decrease in relative levels of $CtBP_L$ in the larva and pupa. Adult levels of $CtBP_L$ remain low in *D. mojavensis*, but recover in *D. virilis*. 50 μg of total soluble protein was loaded on 10% SDS-PAGE and analyzed by immunoblotting with anti-CtBP.

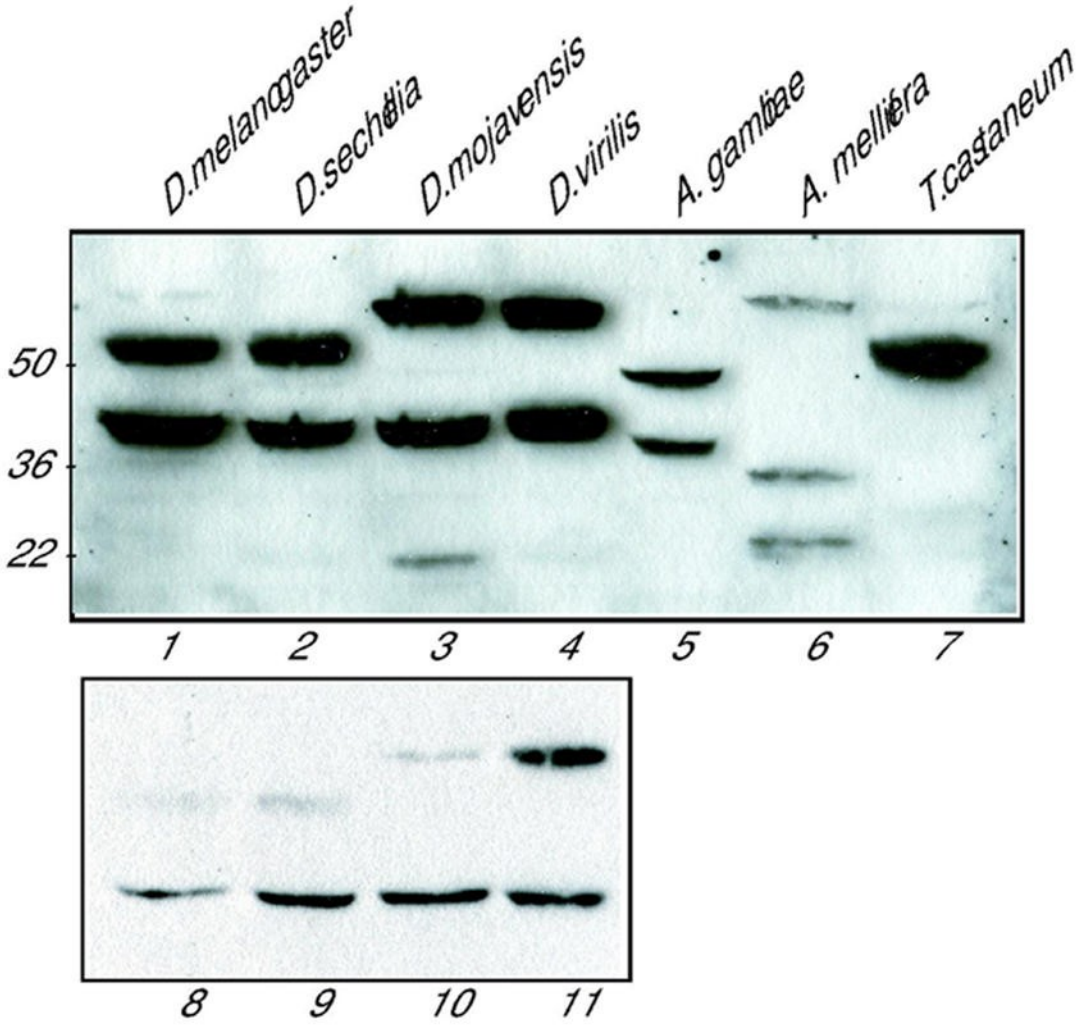

Fig 4. Adult expression of CtBP proteins in four *Drosophila* species, *Anopheles gambiae, Apis mellifera*, and *Tribolium casteneum*

Soluble extracts from adults were analyzed by Western blotting using anti-CtBP. Cross-reacting species similar in size to $CtBP_S$ were noted in all Dipterans. Slower mobility proteins consistent with $CtBP_L$-like species were present in all extracts; multiple bands were detected in extracts from all species except *Tribolium*. The relative abundance of $CtBP_L$ and $CtBP_S$ is masked by the long exposure of the gel; lower panel shows a separate Western blot (lanes 8-11) that was exposed for a shorter time to demonstrate the lower abundance of $CtBP_L$ to $CtBP_S$ in *D. melanogaster*, *D. sechellia*, *and D. mojavensis* adults.