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FUNCTIONAL CHARACTERIZATION OF *Drosophila*
C-TERMINAL BINDING PROTEIN

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Priya Mani

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David Arnosti
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FUNCTIONAL CHARACTERIZATION OF *Drosophila* C-TERMINAL BINDING PROTEIN

By

Priya Mani

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ABSTRACT

FUNCTIONAL CHARACTERIZATION OF *Drosophila*

C-TERMINAL BINDING PROTEIN

By

Priya Mani

Transcriptional repression results from the selective actions of negatively acting repressors and corepressors that function by multiple mechanisms. C-terminal Binding Protein (CtBP) is one such interesting corepressor important for development, oncogenesis and transcriptional regulation. Distinct forms of this protein have been uncovered in vertebrates that are capable of performing a variety of functions, both in the nucleus and cytoplasm. All CtBP isoforms have a high degree of similarity to NAD-dependent hydroxy acid dehydrogenase enzymes, with some isoforms carrying C-terminal extensions (tail). Distinct CtBP proteins are produced in *Drosophila* that differ in the presence or absence of the C-terminal tail. The significance of the homology to metabolic dehydrogenase enzymes and the function of the C-terminal extension is still largely unknown.

Herein we demonstrate that the expression and coding information for distinct CtBP isoforms differing in their C-termini is a conserved feature of phylogenetically divergent organisms. In many *Drosophila* species, the relative levels of isoforms are altered during development with a significant drop in the tailed isoform following embryogenesis. This developmental shift in protein levels can be traced back to a difference in the abundance of

individual spliceform levels and points to the tailed isoform contributing an evolutionarily conserved role.

Intriguingly, all CtBP proteins contain dehydrogenase domains that include an nucleotide (NAD) binding Rossman fold, a putative catalytic core and a broad dimerization region. To investigate the significance of this dehydrogenase homology, mutants in these critical domains were misexpressed in the fly and analyzed for biological activity. Overexpressed wild type isoforms and catalytic mutants resulted in aberrant phenotypes while the NAD binding mutant and the dimerization mutants were completely disrupted for biological activity, with low protein expression. We show that the ability to dimerize is important for CtBP function and the biological activity of the catalytic mutant might be a consequence of dimerization with endogenous CtBP proteins. Our results also indicate that NAD binding is not needed for dimerization and we speculate that it may be required for association with cofactors.

The experiments described here provide a broader understanding of CtBP function in the context of the whole organism, and should in turn facilitate our mechanistic understanding of CtBP function and eukaryotic gene regulation.

To my Dad, Mr. Chandra Shekhar Mani, my Mom, Mrs. Lakshmi Mani,

my husband, Dr. Adwait U. Telang

AND

To my mentor, Dr. David N. Arnosti

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KEY TO ABBREVIATIONS

aa	amino acid
A-P	anterior-posterior
bp	base pair(s)
cDNA:	complementary deoxyribonucleic acid
Co-IP	Co-immunoprecipitation
CtBP	C-terminal Binding Protein
dCtBP	*Drosophila* C-terminal Binding Protein
DNA	Deoxyriboncleic acid
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylene dinitrilo tetra acetic acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMT	Histone methyltransferases
hsp	heat shock promoter
Kb	Kilo base
KDa	Kilo Dalton
NAD	Nicotinamide adenine dinucleotide
NLS	Nuclear localization signal
PAGE	Poly acrylamide gel electrophoresis
PcG	Polycomb
PCR	Polymerase chain reaction
Pol II	RNA Polymerase II
Rb	Retinoblastoma tumor suppressor protein
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
TSA	Trichostatin A
Wt	Wild type

CHAPTER I

INTRODUCTION

Gene expression in eukaryotes is a highly elaborate process under tight regulation at several junctures. An ever-growing body of evidence shows that changes in transcriptional profiles form a vital part of the genetic basis of the evolution of development and contribute to animal diversity (Wray, 2003). Developmental processes are regulated by extensive protein: protein and protein: DNA interactions during the regulation of transcription. Appropriate transcriptional regulation is thus central to cellular homeostasis, and misregulation of gene expression has been implicated in several disease states. Mutations that disrupt developmental processes often map to loci encoding transcription factors, which emphasizes the importance of transcription in development. These regulatory factors are the key to spatio-temporal differential gene expression that must transpire for appropriate cellular differentiation and development to ensue (Lawrence, 1992).

The specificity of differential gene expression in eukaryotes is controlled by the coordinated actions of sequence specific, context-dependent activator and repressor proteins. Early investigations have led to the identification of these coregulators and recent efforts are aimed at the precise characterization of their molecular function. Repressor proteins are aided by corepressor proteins that function by multiple mechanisms and may either help quantitatively by enhancing the overall repression activity (Struffi et al., 2004) or qualitatively, by providing a unique repression activity (Lunyak et al., 2002). Data from several sources suggests that such corepressors link

sequence-specific repressors to the core RNA polymerase II machinery to accomplish direct repression (Austin and Biggin, 1995; Mannervik et al., 1999). In addition, corepressors are also aided by histone modifying activities like deacetylases, methyl transferases and newly-identified demethylases which act cooperatively and are documented to be important players of several repressor assemblies (Arnosti, 2004; Chinnadurai, 2006). An expanding array of post-translational modifications is being acknowledged as means of regulating protein localization and abundance. The ability to use various repression mechanisms in a combinatorial fashion may be exploited to provide unique patterns of gene expression. Current investigations are aimed at defining the precise mechanisms of cofactor function and regulation.

C-terminal **B**inding **P**rotein (CtBP) is a versatile prototype of an expanding family of corepressor proteins that aids in the repression of a diverse array of DNA binding transcriptional repressors in vertebrates and invertebrates alike. In this thesis, I describe studies that analyze the different isoforms of the *Drosophila* homolog of CtBP and reveal dynamic changes occurring in protein levels during development.

Recently, the cellular redox status has also been suggested to influence gene transcription (Imai et al., 2000; Rutter et al., 2001). CtBP proteins resemble NAD-dependent dehydrogenases in structure but whether this similarity extends to function and whether such activity is linked to transcription is unclear. This study also extends to testing the significance of dehydrogenase-like conserved domains within CtBP in the context of a whole organism. These investigations are fundamental to understanding the molecular basis of CtBP's function as a corepressor involved in development.

1.1 CtBP family of proteins

The founding member of the C-terminal Binding Protein (CtBP) family was discovered in 1993 as a 48KDa cellular phosphoprotein that associated with the C-terminal region of the adenoviral 2/5 E1A oncoprotein. This interaction was found to negatively modulate oncogenic transformation, tumorigenesis and metastasis by E1A. Binding was found to depend on a PLDLS (Pro-Leu-Asp-Leu-Ser) motif present near the C-terminal end of E1A protein, conserved among E1A proteins of primate adenoviruses (Boyd et al., 1993; Schaeper et al., 1995). The N-terminal exon of E1A is capable of cellular transformation, but the presence of this motif was shown to restrain exon 1 mediated transformation, suggesting that CtBP binding could influence E1A mediated gene expression (Sollerbrant et al., 1996). Deletion of the CtBP-binding motif abrogated the repressive activity of the C-terminal region. These studies provided early evidence of CtBP acting to antagonize transactivation and led to cloning of the cDNA for human CtBP1 (Schaeper et al., 1995). Shortly thereafter, a highly related human protein termed CtBP2 of 48kDa was identified by BLAST analysis of EST databank sequences. Coincidentally, CtBP2 was simultaneously discovered in a two-hybrid screen using the murine BKLF (Basic Kruppel-like factor) as prey (Turner and Crossley, 2001). Human CtBP1 and CtBP2 are present on different chromosomes and share over 83% amino acid similarity (Katsanis and Fisher, 1998). Other vertebrates such as mice and frogs also have two CtBP homologs, while the invertebrate genomes including those of *C. elegans* and *Drosophila melanogaster* contain a single gene for CtBP. It is in the fly system

that an early role for CtBP in transcriptional repression was established (See Section 1.5). Since then, cell culture based assays and vertebrate models have conclusively shown that CtBP is an essential protein and acts as a bonafide corepressor in these systems.

CtBP is essential for vertebrate development

In mice, both *Ctbp1* and *Ctbp2* transcripts are expressed widely during development in unique and overlapping patterns (Furusawa et al., 1999). To address the *in vivo* significance of multiple CtBP functions, mice carrying mutations in both *CtBP1* and *2* were generated. *Ctbp1*-null mice are reduced in size but viable. In contrast *Ctbp2*-null mice perish by embryonic day 10.5 and show defects in several tissues. This data demonstrates that there are spatial differences between expression patterns of the proteins and suggests that they might have unique functions. CtBP proteins have also been ascribed roles in regulating Golgi dynamics (See Section 1.4), however no Golgi defects in these mutants was noted. Compound mutants for *Ctbp1-/-* and *Ctbp2-/-* arrested at the head fold stage and a reduction in the dosage of one gene enhanced the severity of phenotype associated with the mutation in the other gene (Hildebrand and Soriano, 2002). These results indicate that *CtBP1* and *2* genetically interact. A similar picture emerges from the recent avian expression study of *Ctbp1* and *Ctbp2*, wherein the proteins are expressed in partly overlapping expression domains in the avian embryo (Van Hateren et

al., 2006). This study suggests that avian *Ctbp1* and *Ctbp2* might show functional redundancy in some tissues and specific roles in others.

1.2 Domain structure – Implications for CtBP function

From a structural standpoint, CtBP proteins can be divided into three distinct domains. The first is the substrate-binding cleft that is responsible for specific recognition of PXDLS motifs in binding partners. Co-crystallization of rat CtBP with NAD (H) and a PXDLS like peptide show the presence of this peptide bound to the N-terminus of CtBP. Structural comparisons between binary and ternary complexes reveal that binding of this short peptide is not associated with any major conformational changes in the protein and the peptide binding cleft has no contact with the nucleotide binding region (Nardini et al., 2003).

The second and most conserved of all includes the central nucleotide binding regions. The initial cloning of human CtBP1 and subsequent homology searches revealed extensive homology with NAD-dependent D-isomer specific 2-hydroxy acid dehydrogenases (Schaeper et al., 1995). This homology extends over a nucleotide/NAD binding Rossman fold (GXGXXG) and a putative catalytic histidine residue (towards the C-terminus) in the active site of these enzymes. Dehydrogenase activities have not been previously associated with transcriptional repressors. The structural similarities prompted speculation that CtBP might possess an intrinsic dehydrogenase-like activity that assists in remodeling chromatin structure (Kumar et al., 2002). More

importantly, it also pointed to a role for NAD as a modulator of gene expression. Cellular NAD levels have been linked to transcription before when the Sir2 histone deacetylase (HDAC) was identified as having an NAD-dependent HDAC activity (Imai et al., 2000).

Early attempts to identify dehydrogenase-like activity associated with CtBP were unsuccessful, however a weak activity was reported for CtBP1 (Kumar et al., 2002; Schaeper et al., 1995). Here, Kumar and colleagues tested the dehydrogenase activity of CtBP by combining the reduction of pyruvate to lactate with the oxidation of NADH to NAD+. Recombinant CtBP was able to catalyze this in vitro enzymatic reaction in a dose dependent manner, albeit inefficiently, indicating it is a functional dehydrogenase. The crystal structure for CtBP's minimal dehydrogenase domain revealed a CtBP monomer that forms extensive dimer contacts with another monomer, in the presence of NAD. The dehydrogenase-like domains provide the majority of the contacts needed for dimerization. NAD binds within a cleft at the confluence of two such monomers, inducing conformational changes that allow enhanced binding to transcription factors like E1A. When tested biochemically, mutations in key residues of the dinucleotide binding fold (G181V,G183V, D204A) or the dimerization interface (R141A, R142A, R163A, R171A) disrupt binding to E1A and impair repression by E1A (Kumar et al., 2002). Also conserved in CtBP is the His/Glu/Asp triad that constitutes the active center of dehydrogenases. These residues are conserved in CtBP proteins from vertebrates and invertebrates (See Figure I-1). Mutations in these residues were also shown to compromise the ability of CtBP to contact E1A, and the protein was non-functional when tested as a corepressor.

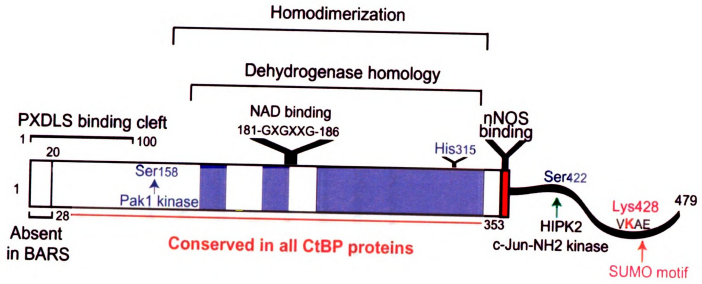
Moreover, this putative catalytic mutant was unable to catalyze the dehydrogenase reaction, unlike the wild type CtBP (Kumar et al., 2002). These observations lend support to the PXDLS binding cleft making direct contacts with the dehydrogenase-like regions.

The structural data described above agrees well with the crystal structure of CtBP3/BARS (a truncated form of rat CtBP1, discussed in Section 1.5). Overall, evidence from both reports concur that dinucleotide binding triggers a closed conformation, triggering closer contacts at the dimerization interface. However, there are some minor differences noted. BARS was co-crystallized was with a short PXDLS-like peptide and the peptide was found to associate exclusively with the N-terminal part of CtBP and binding of the peptide does not induce any noticeable structural alterations (Nardini et al., 2003). This is in contrast to the observations made by Kumar and colleagues for CtBP1, wherein the PXDLS motif is predicted to directly interact with the dehydrogenase-like regions, on the basis of structural data and mutagenesis experiments. Mutations in dehydrogenase-like regions (G172Q in the nucleotide binding region or H304L in the catalytic center) were found to prevent NAD binding, but did not affect binding to transcription factor E1A.

Lastly, the least well-conserved part of the protein is the variable C-terminal extension. This C-terminal tail harbors sites for various post-translational modifications and may play a regulatory role (discussed in Section1.3, See Figure I-1). Vertebrate CtBP proteins all possess C-terminal extensions of ~90 residues that are predicted to be intrinsically unstructured in solution, being composed primarily of disorder promoting residues (56%)

Figure I-1: Key domains of CtBP proteins

CtBP proteins display striking similarity to alpha hydroxy acid dehydrogenases. Regions of highest homology between CtBP and hydroxy-acid dehydrogenases are shown in blue shading. The putative catalytic residue His-315 and the central nucleotide (NAD) binding domain are indicated (Chinnadurai, 2002). The PXDLS binding region has been mapped to the N-terminus of the vertebrate protein (Koipally and Georgopoulos, 2000; Nardini et al., 2003). Residues important for dimerization map within the dehydrogenase domains. CtBP is subject to a host of post-translational modifications, the majority of which have been identified to act on the C-terminal tail of vertebrate CtBP proteins. Phosphorylation sites by Pak1, HIPK2 and c-Jun-NH2 kinase, sumoylation motif, PXDLS binding region and the nNOS-PDZ binding regions are indicated.


Homodimerization
Dehydrogenase homology
PXDLS binding cleft
1
100
20
NAD binding
181-GXGXXG-186
nNOS
binding
His315
Ser158
Pak1 kinase
1
28
353
Ser422
Lys428
VKAE
479
HIPK2
c-Jun-NH2 kinase
Absent
in BARS
Conserved in all CtBP proteins
SUMO motif

(Nardini et al., 2006). Its disordered structure may provide CtBP with the flexibility to contact other proteins that modify this region. It is also plausible that the C-terminus affects CtBP function after recruitment to a transcription factor bearing a PXDLS motif.

1.3 Regulation of CtBP proteins

NAD binding and dimerization

The finding that CtBP-E1A associations and dimerization are regulated by NAD suggest that this cofactor can influence transcriptional outputs. Structural data from CtBP proteins show that small metabolic intermediates like NAD+ and NADH can bind and induce conformational changes that have functional consequences relating to repression (Kumar et al., 2002). Although the effect of dinucleotide binding on CtBP function has been investigated by many, the differential efficacy of NAD+ (oxidized) v/s NADH (reduced) is debatable. Goodman and colleagues speculate that since free NAD+ levels exceed free NADH levels in a cell, changes in the redox state could be manifested by NADH levels that are limiting. Consistently, NADH was found to efficiently enhance vertebrate CtBP-E1A binding three orders of magnitude better than NAD+ (Zhang et al., 2002). Another study measuring binding affinities shows CtBP to have higher affinity for NADH than NAD+ (>100 fold) (Fjeld et al., 2003), however other studies find NAD+/NADH to be equally effective in enhancing CtBP binding E1A (Kumar et al., 2002). Thus, a

speculative model has been suggested for CtBP to play the part of a cellular redox sensor that interprets the cellular metabolic demands and accordingly directs transcription (Zhang et al., 2002). Binding of dinucleotide is thought to bring about conformational changes in protein structure that stimulate dimerization (Balasubramanian et al., 2003). This might be one mechanism by which CtBP proteins enter the nucleus and regulate target genes. In this manner, NAD can be considered a switch that assembles the CtBP complex.

A good example of the impact of NAD on CtBP activity is a recent study describing CtBP to be a corepressor for neuronal genes such as those involved in drug-resistant epilepsy. CtBP was recruited by NRSF (neuronal restrictive silencing factor) and was found to enhance NRSF-dependent repression. The CtBP-NRSF direct interaction was disrupted upon increasing levels of NADH (by speeding up glycolysis) resulting in de-repression of neuronal targets. Conversely, the glycolytic inhibitor 2DG (2-deoxy-D-glucose) was found to enhance NRSF directed repression of target genes, that showed a marked reduction in levels of H3-K9 acetylation (mark of activation) and an increase in H3-K9 methylation (mark of repression). Repression was lost in CtBP null mouse embryonic fibroblasts (MEFs) (Garriga-Canut et al., 2006). This demonstration of small molecule regulation of energy metabolism directly impacting chromatin structure and gene expression in disease states is novel, but altering central processes like glycolysis is very likely to cause pleiotropic effects.

Multiple CtBP proteins are encoded by multifunctional genes

In mammals, the CtBP family is encoded by two genes: *Ctbp1* and *Ctbp2*. Both genes generate multiple isoforms by either alternative splicing or differential promoter utilization (See Figure I-2). *Ctbp1* undergoes alternative splicing at the N-terminus to yield two splice variants: CtBP1-L (Long et al.) and CtBP1-S (short) that differ by the presence of 11aa additional at the N-terminus of CtBP1-L. Differential promoter usage and gene splicing from *Ctbp 2* generates CtBP2 and a retinal-specific variant, RIBEYE (Chinnadurai, 2002). RIBEYE protein is expressed from a tissue specific promoter located within an intron in *Ctbp2* that results in a large (~ 565 aa, also called the A domain) N-terminal domain being fused to CtBP2 except for the 20 aa from the N-terminus (Schmitz et al., 2000). CtBP2 uses an upstream promoter and alternative splicing to eliminate the exon that encodes the N-terminal domain of RIBEYE.

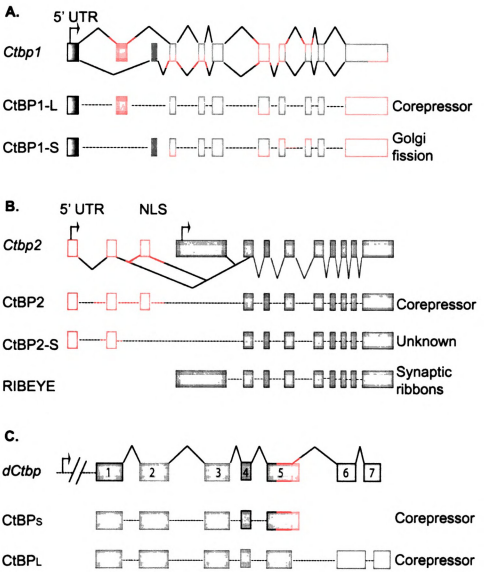
CtBP1-L and CtBP2 have been deemed corepressors, while CtBP1-S has been ascribed a unique cytoplasmic role in Golgi fissioning. CtBP1-L has been found to associate with synaptic ribbons and its roles in the cytoplasm are speculative. The RIBEYE variant of CtBP2 is found in abundance in specialized photoreceptor ribbon synapses involved in vision, and may be involved in regulating neurotransmitter release (Schmitz et al., 2000). Interestingly, the exon encoding the RIBEYE specific region is absent in worm and fly genomes, suggesting it is a vertebrate novelty. A recent study also reported the identification of a splice variant of CtBP2 that is localized

Figure I-2: CtBP genomic structure and splicing patterns

A. Alternative splicing of the vertebrate CtBP1 gene generates CtBP1-L and CtBP1-S possessing divergent functions. Exons exclusive to CtBP1-L (red) and CtBP1-S (blue) are color coded.

B. Alternative promoter utilization and gene splicing create CtBP2 that includes an NLS (yellow box) while RIBEYE is produced from an intronic promoter that encodes a large A domain fused to the remainder of the CtBP2 coding sequence. RIBEYE hence lacks the NLS. CtBP2-S is a splice variant of CtBP2 that is devoid of the NLS and thus predominantly cytosolic.

C. *Drosophila melanogaster* CtBP is spliced to produce $CtBP_L$ and $CtBP_S$ that differ by the presence of the C-terminal extension encoded by exons 6 and 7 (numbered orange boxes), exclusive to $CtBP_L$.
Cartoons are not drawn to scale and are adapted from (Verger et al., 2006).


A.
5' UTR
Ctbp1
CtBP1-L
Corepressor
CtBP1-S
Golgi fission
B.
5' UTR
NLS
Ctbp2
CtBP2
Corepressor
CtBP2-S
Unknown
RIBEYE
Synaptic ribbons
C.
dCtbp
1
2
3
4
5
6
7
CtBPs
Corepressor
CtBPL
Corepressor

specifically to the cytoplasm (See Figure I-2 and Section 1.4) (Verger et al., 2006). The *Drosophila* homolog of *CtBP* is also subject to alternative splicing and generates two major isoforms (See Figure I-2) as discussed in detail in Section 1.5 and Chapter 2.

PXDLS interactions: Significance and determinants of binding

Early studies with the E1A protein suggested that CtBP is recruited to promoters through interactions with a PXDLS motif (Pro-Leu-Asp-Leu-Ser in E1A)), where X is usually a bulky group like Leucine, Iso-leucine or Valine. This has been found true for dozens of transcriptional effectors containing PXDLS-like motifs that bind CtBP. These include zinc-finger proteins like Ikaros (Perdomo and Crossley, 2002), BKLF (van Vliet et al., 2000) , FOG, Snail, delta EF1 (Furusawa et al., 1999), Evi-1 (Palmer et al., 2001), bHLH group proteins like Hairy (Poortinga et al., 1998) and viral oncoproteins like E1A. Subsequent studies confirmed that a vast majority of DNA binding transcription factors that need CtBP contained variants of this motif, some in more than one copy. Mutation in this short stretch of residues has been tested in many proteins and has been found to abolish CtBP interaction and repression (Boyd et al., 1993; Keller et al., 2000; Vo et al., 2001). Most notable is a study describing a naturally occurring mutation in the PXDLS in human homeodomain protein, TGIF. As a consequence of this mutation, TGIF-CtBP interaction is disrupted, resulting in holoprosencephaly and craniofacial abnormalities (Melhuish and Wotton, 2000). In *Xenopus*, the

hematopoietic transcription factor FOG represses erythroid development by the recruitment of CtBP via a PXDLS motif. Mutations in this motif are seen to abrogate the interaction. These results suggest that CtBP plays an important role in hematopoiesis (Deconinck et al., 2000) .

CtBPs also bind transcription factors that do not contain an identifiable PXDLS. Many of the vertebrate effectors like HDACs (HDAC1, 2 and 5) fall into this category. This is also true for the *Drosophila* HDAC Rpd3 that does not have an discernible dCtBP binding motif but is a part of the Knirps repressive complex that includes CtBP, and interacts physically and functionally with Knirps (Struffi and Arnosti, 2005). CtBP physically interacts with several histone acetyltransferase (HAT) enzymes whose bromodomains contain a consensus PXDLS like motif. However, deletions of the bromodomain or mutations in the PXDLS within the bromodomain retain interaction with CtBP, suggesting that additional non-canonical sites must exist (Senyuk et al., 2005).

Each unit of CtBP harbors a PXDLS binding cleft, suggesting that dimers can serve to bridge two PXDLS partners together. When fused to a heterologous DNA binding domain, disruption of the PXDLS responsive cleft in CtBP does not affect its repression activity. In contrast, repression is abolished when tested in the absence of the heterologous domain, suggesting that the cleft does not determine association with other effectors, but primarily functions to localize CtBP to repressors bound at target promoters (Quinlan et al., 2006).

In the case of many transcription factors, it has been suggested that neighboring residues flanking the PXDLS motif might contribute to the folding

capacity of this region and consequently affect its interaction with CtBP. Structures of synthetic peptides examined using NMR spectroscopy suggest that peptides have different affinities for different CtBPs e.g. the *Drosophila* Hairy protein binds *Drosophila* CtBP stronger than human CtBP suggesting alternative CtBPs have distinct binding specificities (Molloy et al., 2001). In E1A protein, acetylation of a lysine residue close to the PXDLS motif in the C-terminus disrupts binding to both CtBP1 and 2 when tested as a synthetic peptide. This interference could be through structural changes, charge neutralization or a change in the subcellular localization of CtBP (Molloy et al., 2006).

Mechanisms dictating Localization and Stability of CtBP proteins

CtBP1 and 2 are highly similar proteins and possess similar transcriptional repression activities, although new evidence suggests that their activities can be differentially regulated, by specific control of their subcellular location. CtBP proteins have been detected both in the nucleus and in the cytoplasm. Much of our current knowledge of CtBP localization is derived from studies centered on CtBP1-L, whose cellular location is actively regulated. CtBP1-L is predominantly cytoplasmic in Cos cells but when coexpressed with a PXDLS containing protein, such as Net or HIC1 transcription factors, it is relocated into the nucleus (Criqui-Filipe et al., 1999; Deltour et al., 2002). CtBP1-L has been shown to interact with the PDZ domain (protein-protein interaction domain; *P*SD-95, *d*iscs-large and *z*ona

occludens-1) of nNOS (neuronal nitric oxide synthase) via a C-terminal DXL sequence, which shuttles CtBP from the nucleus to the cytoplasm. This mechanism might also potentially serve to make CtBP available for its cytoplasmic roles as discussed in Section 1.5.

Posttranslational modifications are also accountable for CtBP subcellular distribution. CtBP1-L is SUMOylated at Lys 428 via PC2 (Polycomb group protein) and this modification has profound effects on its distribution into the nucleus, antagonistic to that of nNOS. Mutation of Lys 428 into Arg 428 shifts CtBP from the nucleus to the cytoplasm and consistent with its localization, restricts CtBP from acting as a corepressor (Lin et al., 2003). CtBP2 lacks this SUMOylation site and the PDZ binding domain and is likely to be regulated differently.

Independent phosphorylation of CtBP by three different kinases also plays a regulatory role. The p21-activated kinase (Pak1) has been shown to phosphorylate CtBP1-L on Ser 158, a modification that relocates CtBP into the cytoplasm similar to nNOS activity (Barnes et al., 2003). Homeodomain Interacting Protein kinase 2 (HIPK2) is a serine/threonine kinase involved in transcription and apoptosis. HIPK2 targets Ser 422 in the C-terminus of CtBP for phosphorylation. HIPK2 has previously been shown to phosphorylate p53 in response to UV stress, thereby promoting apoptosis by induction of genes like *Bax*, *PERP*, *p21* and *Noxa* which are also upregulated in CtBP knockout MEFs (Grooteclaes et al., 2003). HIPK2 modification marks CtBP for UV triggered ubiquitination and subsequent degradation via the proteasome providing an alternative pathway for apoptosis, by a p53 independent mechanism (Zhang et al., 2005). c-Jun-NH2 terminal kinase is the third

kinase that also targets Ser 422 for phosphorylation and degradation. C-jun-NH2 kinase plays important roles in inducing apoptosis under cellular stress conditions like UV irradiation or cisplatin treatment (Wang et al., 2006). Under both these conditions, CtBP levels were markedly reduced. These observations indicate Ser 422 to be a mark for CtBP proteasomal degradation and lead to the speculation that CtBP may respond to a wider network of signals than those currently known.

Amongst the CtBP proteins, CtBP1-S and RIBEYE appear to have roles in the cytosol, CtBP2 is predominantly nuclear and CtBP1-L is both nuclear and cytoplasmic. A search for regions that serve as signals for localization within CtBP1-L and CtBP2 identified a nuclear localization signal (NLS) within CtBP2 (Verger et al., 2006). This 'KRQR' sequence is present in the first exon of CtBP2 and is critical for its nuclear distribution. This motif is not present in CtBP1-L. Within this unique CtBP2 NLS, mutagenesis data indicates that the Lys 10 and Arg 11 are critical for localization. Interestingly this region is conserved in the *Drosophila* CtBP protein. This result is consistent with reports that describe CtBP2 as a nuclear protein (Chinnadurai, 2006). When cotransfected with other CtBP proteins, the NLS of CtBP2 is capable of shuttling the other CtBP proteins into the nucleus by virtue of dimerization. A splice isoform of CtBP2 that lacks this NLS (CtBP2-S) localizes to the cytoplasm. In contrast, a parallel study suggests that instead of functioning as a classical NLS, the Lysine 10 residue might be a site for acetylation of CtBP by p300 that directs it to the nucleus. Using a non-acetylatable version of K10R, this study demonstrated that this acetylation is required for nuclear retention of CtBP2 (Zhao et al., 2006). Further studies are

needed to distinguish whether the lysine 10 residue in CtBP2 plays a role in nuclear import or nuclear retention. This mode of regulation of CtBP proteins might have important ramifications on their roles in transcription, development and cellular differentiation.

1.4 Mechanism of CtBP mediated transcriptional repression

CtBP is recruited to target promoters by DNA-binding repressors, but details of events that follow are unknown. The precise nature of CtBP's mode of repression is a subject of intense investigation. Human CtBP1 associates with several HDACs and histone deacetylation might be a major contributor to repression (Sundqvist et al., 1998). However, CtBP mediated repression has been reported to be insensitive to the Class I HDAC inhibitor, Trichostatin A, signifying that HDAC-independent mechanisms might be at play (Ryu and Arnosti, 2003). A first step was taken at identifying these cofactors with the biochemical purification of vertebrate CtBP1 in HeLa cells. The associated proteins were: DNA binding proteins like ZEB1, histone modifiers like histone deacetylases and methyltransferases (HDACs 1 and 2, related HMTs like EuHMT1 and G9a), chromodomain containing protein like HPC2 (human polycomb), Co-REST and related proteins (corepressor for REST transcription factor) and NPAO (nuclear polyamine oxidase). CtBP2 was also found in the same complex, supporting the notion that CtBP proteins can heterodimerize. This core CtBP complex could efficiently cause H3K9 and H3K27 methylation of core histones, both of which are marks of repression. These modifications

were also enriched at a CtBP target, *E-cadherin*. siRNA targeting CtBP not only reduced occupancy of CtBP and EuHMT1 on the *E-cad* promoter but also decreased methylation of H3 (Shi et al., 2003). Studies on the enzymatic activity of NPAO led to the identification and characterization of the histone demethylase, LSD1 (lysine specific demethylase) (Shi et al., 2005). LSD1 specifically demethylates H3K4 (a mark of active chromatin) via an amine oxidation reaction (Shi et al., 2004). Its presence in the CtBP complex suggests that CtBP proteins co-ordinate distinct enzymatic events that convert active chromatin to a repressed state.

Another recently characterized mechanism of CtBP repression includes physical interactions with HAT coactivators like p300 and CBP that contain PXDLS motifs. CtBP was shown to specifically impair the HAT function of these proteins by suppressing H3 acetylation (K9, K14 and K18) in an NADH dependent manner (Kim et al., 2005; Senyuk et al., 2005). The association of CtBP with both coactivators and corepressors of transcription suggests that diverse CtBP complexes with distinct transcriptional properties might exist *in vivo*.

In humans, CtBP also interacts with CtIP (C-terminal Interacting Protein). CtIP bridges CtBP to Retinoblastoma protein, human Polycomb and BRCA1 (breast cancer tumor suppressor) (Li et al., 1999; Meloni et al., 1999; Sewalt et al., 1999). CtBP proteins also interact with the Polycomb protein (Pc) in *Xenopus* (Sewalt et al., 1999). A functional role for this interaction has already been discussed with regard to the localization of CtBP proteins by SUMOylation. The Polycomb complex isolated from *Drosophila* does not appear to contain CtBP and a CtIP-like protein is yet to be identified (Levine et

al., 2002). These observations postulate that there might be key differences in the complexes formed by human CtBP1 vs. *Drosophila* CtBP.

CtBP proteins may act mostly as bridging molecules to recruit the above-described activities to the DNA binding repressor. As discussed above, CtBP itself has an inherent dehydrogenase activity that might partake in repression, but this remains unproven. Dehydrogenases have not yet been documented to possess repressive activities, however an Oct-1 transcription complex was recently found to utilize a dehydrogenase enzyme for the activation of the histone H2B promoter (Zheng et al., 2003).

1.5 A distinct cytosolic role ascribed to CtBP

In a study of factors that regulate membrane tubulation, CtBP3 was identified as a protein that is ribosylated in rat kidney cells upon treatment with the fungal toxin Brefeldin A (Nardini et al., 2003; Spano et al., 1999). CtBP3 displays 97% identity to human and mouse CtBP1-L and 79% identity to CtBP2. CtBP3 lacks the first 11 aa encoded by CtBP1; whether this protein represents the rat version of CtBP1 or an N-terminal splice variant of CtBP1 is not certain. The current consensus in the field is that CtBP3 represents an N-terminally truncated version of CtBP1. This protein is termed CtBP1-S or BARS (Brefeldin-A ribosylated substrate). Recombinant BARS has been shown to possess a weak acyl transferase enzymatic activity that catalyzes acylation of lysophosphatidic acid to phosphatidic acid (a lipid involved in membrane fissioning) (Weigert et al., 1999). This coincides with the onset of

mitosis that requires organelle partitioning between daughter cells. This activity of BARS is thought to lead to accumulation of phosphatidic acid in the Golgi membrane, enhancing the curvature of the phospholipid bilayer and thereby causing disassembly of the Golgi network (Corda et al., 2006). To test the role of BARS in this process, BARS activity was reduced in rat kidney cells using dominant negative mutants or by mRNA depletion using antisense oligos. These treatments prevented Golgi partitioning and arrested the cell cycle at G2. Both Golgi fragmentation and the onset of cell cycle were restored upon addition of recombinant BARS (Hidalgo Carcedo et al., 2004).

In support of this data, another study found that in mitotic cells, human CtBP becomes associated with centrosomes during mitosis. Golgi morphogenesis is initiated and regulated within centrosomes, implicating CtBP in this process (Spyer and Allday, 2006). A more recent study re-evaluated the acyl transferase activity associated with BARS and claimed it to be a contaminant associated with the recombinant BARS protein (Gallop et al., 2005). Intriguingly, dual *Ctbp1* and *Ctbp2* mouse knockouts are embryonic lethal but no Golgi defects are observed and MEFs can be cultured from these animals with no visible Golgi defects. Clearly, much remains to be proven concerning the role of CtBP in Golgi fission.

1.6 CtBP as a corepressor during *Drosophila* development

The *Drosophila* blastoderm embryo serves as a premier model for the study of eukaryotic transcriptional regulation, providing an amenable background for the study of both cis- and trans-regulatory elements. Early embryonic development in *Drosophila* is controlled by a cascade of genes that encode for transcription factors. The cascade is turned on by preformed mRNAs and proteins that are synthesized by the mother and deposited in the egg prior to fertilization. The maternally derived proteins set up spatially distributed morphogenetic gradients that establish the antero-posterior (A-P) and dorso-ventral axes. These morphogen proteins can then activate or repress zygotic genes at specific positions along both axes and thereby specify patterning along the embryo (St Johnston and Nusslein-Volhard, 1992).

Gap genes are the first category of zygotically transcribed genes, expressed in broad expression domains. They were identified based on phenotypes in a genetic screen looking for mutants lacking large sections of abdominal segments along the A-P axis (Nusslein-Volhard and Wieschaus, 1980). Gap genes encode well-characterized repressor proteins whose primary function is to refine the patterns of pair-rule genes, the next class of genes in the segmentation cascade. Their repression activity is described as short-range, acting at distances of up to 100-150 bp to inhibit the basal promoter or upstream bound activators (Gray and Levine, 1996; Nibu et al., 2003). This is in sharp contrast to long-range repressors that globally suppress transcription over several kilobases (Cai et al., 1996). Short-range

repressors employ CtBP as a corepressor, while long-range repressors use the Groucho corepressor. It has been long believed that Groucho and CtBP represent two distinct pathways of repression that form the basis for this so called 'range' of repressor activity.

Pair-rule genes are expressed in seven transverse stripes along the anterior-posterior axis and in turn, they control the fourteen-stripe pattern of the subsequently expressed segment polarity genes that delineate the future segments of the fly. Identification of these zygotic gene products involved in body plan determination of a *Drosophila* embryo is the outcome of large genetic screens that searched for embryonic lethal mutations.

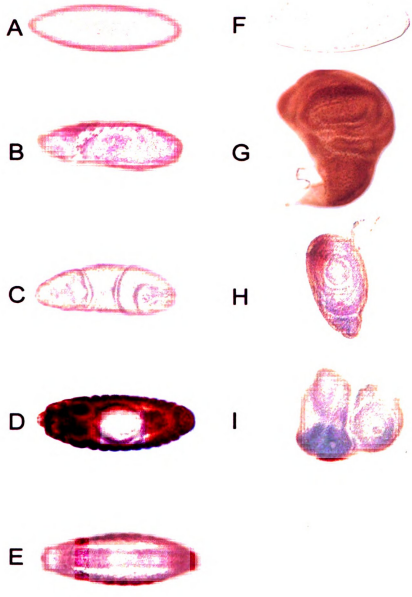
CtBP plays a vital role in this hierarchical organization by contributing to the repression abilities of short-range repressors encoded by gap genes. The first thread of evidence linking CtBP to short-range repression came from yeast two-hybrid assays, in which Knirps and Snail, two short-range repressors were used as bait to screen an embryonic cDNA library (Nibu et al., 1998b). Both proteins selected *Drosophila* CtBP cDNA, specifying a putative protein of 383 residues and exhibiting high sequence similarity with the human CtBP. The CtBP gene is expressed during early oogenensis and the message is deposited in the egg prior to fertilization. Following zygotic induction, CtBP proteins can be traced throughout development to be ubiquitously and uniformly expressed in the fly (See Figure I-3).

It has been demonstrated that CtBP is essential for short-range transcription during development by analyzing embryos lacking maternal CtBP. dCtBP is encoded by a single gene whose locus has been mapped cytologically to 87D8-9 on the third chromosome. P1590 is a homozygous

lethal P-element insertion within the CtBP coding region that results in homozygotes dying as pharate adults (i.e. still within pupal case). Mutant embryos containing reduced CtBP levels demonstrate a host of patterning defects that can be attributed to impaired activity of short-range repressor proteins like Knirps, Krüppel and Giant. In situ hybridization assays show that the expression of these repressors is mostly normal in CtBP mutants, but the expression of their pair-rule targets such as *even-skipped* and *fushi-tarazu* is severely disturbed (Nibu et al., 1998a). Like vertebrate repressors, short-range repressors in *Drosophila* recruit CtBP via a PXDLS motif. Mutations in this motif compromise the activity of these proteins, indicating that CtBP-dependent repression represents a major form of transcriptional regulation during development. A direct transcriptional repression activity of dCtBP has also been shown by Gal4-CtBP in a tethering assay wherein CtBP can repress nearby transcriptional activators but not the basal promoter elements (Nibu et al., 1998b; Sutrias-Grau and Arnosti, 2004). This tethering assay has been exploited further to question the relevance of CtBP's homology to dehydrogenases. Mutations made in the putative catalytic histidine are found not to compromise CtBP corepressor function while mutations in the nucleotide-binding fold destroy its ability to obstruct repression on integrated reporter genes (Sutrias-Grau and Arnosti, 2004). A protein interaction map in *Drosophila* emphasizes that CtBP forms a major transcriptional regulatory

Figure I-3: *Drosophila* CtBP proteins are ubiquitously distributed during development

Stage specific embryos were collected and imaginal discs were harvested from third instar larvae. Samples were fixed in a heptane-formaldehyde solution as per lab protocol and immunostained with anti-CtBP rabbit polyclonal serum (1:500) (F) embryo stained with rabbit preimmune, (B-E) embryos from progressive stages during development, (G-I) wing, eye and leg imaginal discs extracted from late third instar larvae (P.Mani, unpublished).


A
B
C
D
E
F
G
H
I

network where each interactor contains a discernible P-DLS like motif (Giot et al., 2003).

CtBP and long-range repression by Hairy

As typified by the Hairy protein in *Drosophila*, long-range repressors can function over many kilobases to block transcription, in sharp contrast to short-range repressors. The mechanisms underlying these modes of repression are unclear (Courey and Jia, 2001). Possible clues have arisen from the observations that short-range repressors mediate repression by the recruitment of CtBP, while long-range repressors recruit a different corepressor, Groucho. These corepressor may mediate repression by distinct pathways (Chen and Courey, 2000). However, both CtBP and Groucho have been proposed to utilize chromatin remodeling mechanisms through the recruitment of histone deacetylases like Rpd3 (Chen et al., 1999; Struffi and Arnosti, 2005). The long-range repressor Hairy also shows a weak interaction with CtBP and has a canonical CtBP binding motif. The removal of the weak dCtBP interaction motif (PLSLV) does not impair Hairy-mediated repression of its targets. Instead, removal of this motif augments Hairy function. CtBP and Groucho binding to Hairy may be antagonistic because just nine amino acid residues separate the CtBP binding P-SLV-K and Groucho binding WRPW motifs. Mechanistically speaking, when dCtBP and Groucho both bind, they might be unable to interact with additional corepressors or contact their target proteins in the core transcription complex (Zhang and Levine, 1999). Such antagonism is supported by genetic studies, which suggest that lowering the

dose of maternal *dCtBP* products can partially suppress the embryonic phenotypes of *hairy* mutants while reducing *groucho* can enhance it (Phippen et al., 2000; Poortinga et al., 1998).

The simplest interpretation of these observations is that the dCtBP and Groucho interfere with one another when both are bound to Hairy, but the rules governing the choice of cofactor recruited are undetermined. Another cofactor of Hairy is the Sir2 protein that encodes an NAD+ -dependent histone deacetylase. A global chromatin profiling study looking at genomic recruitment of Hairy and its cofactors demonstrates that while Groucho is believed to be the major cofactor, it is found to be associated with only subsets of Hairy targets. Surprisingly, CtBP and Sir2 are predominantly found at Hairy targets with their association being largely overlapping and distinct from Groucho (Bianchi-Frias et al., 2004).

CtBP as a corepressor for signaling pathways in the fly

Numerous signaling pathways are seen to converge upon general transcription coregulators. *Decapentaplegic (dpp)* encodes a TGF-β homolog that functions as a long-range signaling morphogen, specifying cell fates in a dose-dependent manner. A response to thresholds of Dpp proteins is regulated by an opposing gradient of Brinker, which is a *dpp*-target and a transcriptional repressor. Brinker's repression domain contains binding sites for both CtBP and Groucho corepressors and physical interactions have been established. For Brinker to silence its endogenous targets, Groucho alone is

sufficient. For simpler targets such as Brinker autoregulation, both corepressors can function interchangeably and adequately. Like Hairy, in the absence of Groucho, Brinker's repressive capacity is impaired and unlike Hairy, CtBP's absence also seems to affect Brinker output to a smaller extent (Hasson et al., 2001). These results suggest that Brinker uses multiple modes of repression (provided by CtBP and Groucho) that provide it with a flexibility to silence a variety of genes in response to a gradation in Dpp activity during development.

The Notch signaling pathway is employed in a variety of cell fate decisions during development. The Notch signal is transduced via Su(H) [Suppressor of Hairless] that acts as a repressor in the absence of Notch signal and repression is critical for appropriate cell fate specification. In the presence of Notch signaling, Su(H) is an activator. Hairless (H) acts as a corepressor for Su(H) by antagonizing its activation via the recruitment of CtBP and Groucho. Reduction in levels of either CtBP or Groucho enhances Hairless loss of function phenotypes, and mutating Hairless's CtBP binding motif debilitates its activity, suggesting that both corepressors are vital components of this signaling pathway (Barolo and Posakony, 2002; Barolo et al., 2002).

Regulation of the Wnt/ β-catenin signaling cascade is also utilized in cell fate determination and oncogenesis. Constitutive induction of this pathway is observed in many cancers, especially in colorectal carcinoma. In the absence of Wnt signal, TCFs (T-cell factor) function as repressors of Wnt target genes, similar to the functionings of the Notch pathway. In *Xenopus*, xCtBP is known to act as a corepressor for xTCF-3 (Brannon et al., 1999). In

the fly, TCF's are thought to recruit Groucho for repression and when Wnt signaling is on, binding of β-catenin displaces Groucho and repression is relieved. CtBP can bind to vertebrate TCF's, and CtBP overexpression inhibits TCF mediated activation of Wnt targets, but no direct interaction of TCF and CtBP has been shown. One model suggests that CtBP antagonizes Wnt pathway by binding to APC tumor suppressor (Adenomatous polyposis coli) that causes it to sequester β-catenin away from TCFs (Hamada and Bienz, 2004). Some support for this model comes from a study where APC and CtBP can be traced on a Wnt target and this coincides with a loss of β-catenin from TCFs (Sierra et al., 2006). As colorectal cancer cells all have mutations in APC proteins, the functional interaction of APC and CtBP suggests a role for CtBP as a tumor suppressor. In contrast, another model suggests CtBP to be recruited by an unknown DNA binding protein and act in parallel to TCF to repress Wnt targets (Fang et al., 2006).

CtBP- independent repression activity

Multiple repression activities may assist quantitatively or qualitatively in regulating gene expression. Three general mechanisms of transcriptional repression in eukaryotes include competition, where repressors directly compete with activators for a common cis-element, and blocking the basal transcriptional machinery or direct repression. The third is by quenching, whereby repressors repress activators co-occupying adjacent sites. Chromatin modifications that reduce accessibility of transcription factors to the

DNA template may involve covalent modifications of histones or DNA (Strahl and Allis, 2000). In addition, cis-acting elements named silencers or boundary elements may prevent transcription of a gene by preventing crosstalk when located between a promoter and enhancer (Gerasimova and Corces, 2001).

Embryo repression assays performed with short-range repressors Knirps, Giant and Krüppel all provide basis for CtBP-dependent and CtBP-independent modes of repression. Knirps has a repression domain towards the C-terminus that encodes a PXDLS like motif important to bind CtBP. In addition, an N-terminal region has also been mapped that is active for repression and does not contain any canonical CtBP binding motifs. This activity is evident in experiments in which the CtBP corepressor is absent. In transgenic embryos containing integrated reporters and lacking CtBP, Knirps can still repress its endogenous target, the *eve* stripe 3 enhancer (Keller et al., 2000).

In case of Krüppel, when its binding sites do not overlap but are adjacent to activator binding sites, repression is dependent on CtBP by quenching. Quenching might involve hindering activator-basal machinery crosstalk or it might invoke the intrinsic dehydrogenase activity ascribed to CtBP to remodel chromatin. However, Krüppel can repress by competition without any help from CtBP, when the activator sites overlap with Krüppel repressor binding sites, indicating a CtBP-independent mode of repression (Nibu et al., 2003). Giant is also able to effectively regulate *hunchback* while its other targets like *eve* stripe 2 are derepressed in a CtBP mutant background proposing that Giant too can function via CtBP-independent pathways (Strunk et al., 2001). The relevance of these multiple activities was

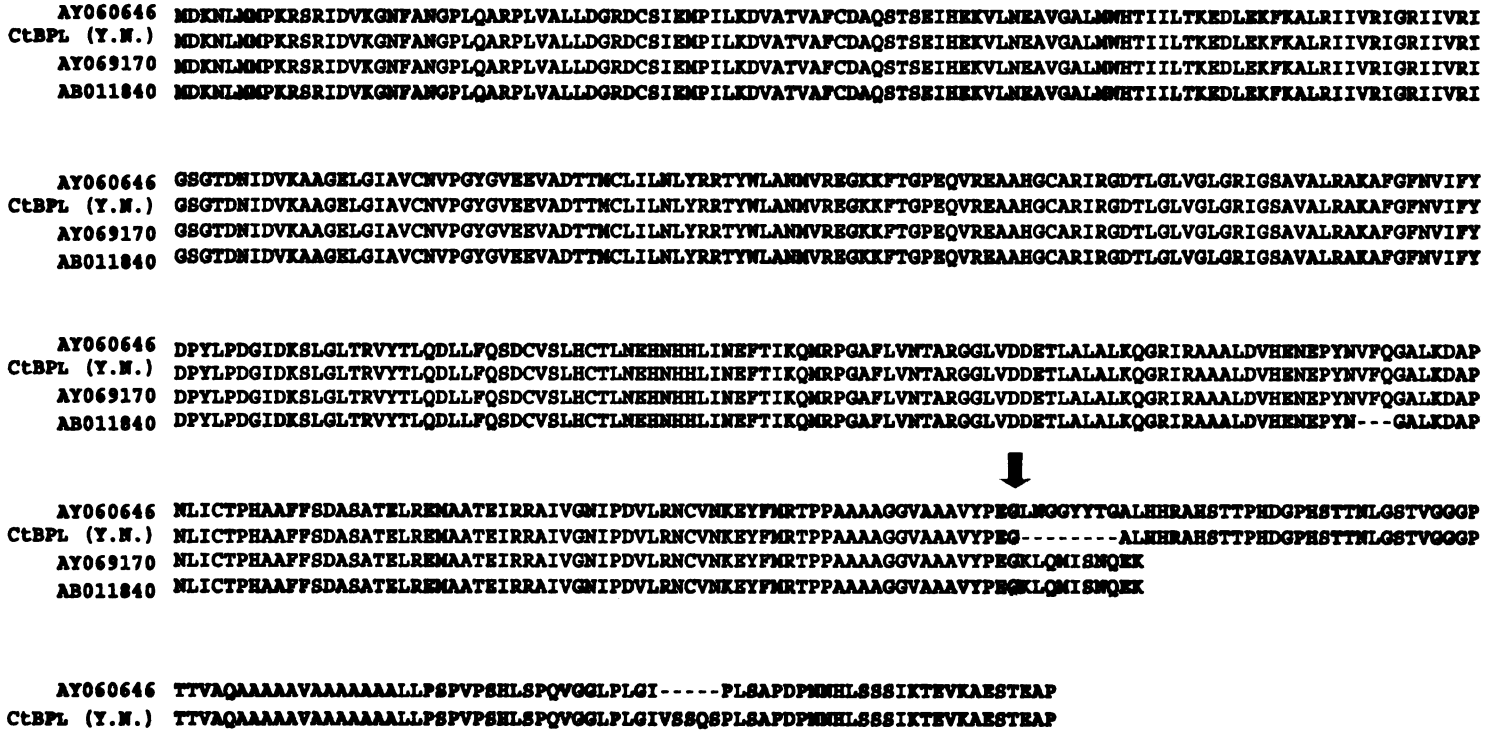
put to test in the case of Knirps. It was found that supplying CtBP-independent activity at higher than regular levels could repress a target that normally requires CtBP, suggesting that CtBP contributes in a quantitative fashion to boost repressor output (Struffi et al., 2004).

1.7 Alternative splicing of Drosophila CtBP

In the fly embryo, multiple CtBP transcripts are expressed ubiquitously throughout development that encode polypeptides of 383, 386, 476 and 479 amino acids (See Figure I-4). The first 376 amino acids required for interaction with short-range repressors are common to all four isoforms (essentially composed of only the dehydrogenase-like domain). Any distinctions in the function of individual isoforms is yet unidentified. These distinct isoforms are produced from alternative splicing that use alternative donor / acceptor sites and differ largely in the presence or absence of a C-terminal tail of ~90 amino acids of unidentified significance. The 476 and 479 aa isoforms both include the C-terminal tail and differ in the alternate use of the splice acceptor that results in the presence/absence of an 'LNGGYYT' motif at the start of exon 6 and a 'VSSQS' motif at the start of exon 7 (Also see Chapter II). Both the 383 and 386 aa isoforms lack the C-terminal extension and differ in the inclusion of a 'VFQ' motif by resorting to different splice acceptors at the start of exon 5. It is very likely that these different isoforms might function in a tissue or stage specific manner with

Figure I-4: Alignment of different CtBP isoforms generated by alternative splicing in *Drosophila*

The *Drosophila CtBP* gene encodes differentially spliced products that are divergent at their COOH-terminal regions (conserved residues are shaded in yellow). Two isoforms [AY060646 (479aa) and NM_001014617 (476 aa)] include an unstructured C-terminal extension of ~90 aa of unknown function and differ in the alternative use of a splice donor that results in the inclusion of 'LNGGYYT' motif in the 479 aa isoform and an inclusion of a 'VSSQS' motif in the 476 aa isoform. The two shorter isoforms [AY069170 and AB011840] are essentially composed of just the dehydrogenase-like domains and do not include the C-terminal tail. These also differ in the use of a splice donor that results in the inclusion of a 'VFQ' motif in AY069170. The red arrow indicates a conserved splice donor that results in the generation of the longer isoforms. This splice donor represented by a conserved glycine codon within exon 5 that is not subject to silent mutations in several organisms, supporting the notion that isoforms with tails are functionally important.
The experiments in Chapter II, III and Appendix A were performed using the 479 and 383 aa isoforms obtained from Yutaka Nibu, Weill Medical College of Cornell University, New York.


AY060646 MDKNLMMPKRSRIDVKGNFANGPLQARPLVALLDGRDCSIEMPILKDVATVAFCDAQSTSEIHEKVLNEAVGALMWHTIILTKEDLEKFKALRIIVRIGRIIVRI
CtBPL (Y.M.) MDKNLMMPKRSRIDVKGNFANGPLQARPLVALLDGRDCSIEMPILKDVATVAFCDAQSTSEIHEKVLNEAVGALMWHTIILTKEDLEKFKALRIIVRIGRIIVRI
AY069170 MDKNLMMPKRSRIDVKGNFANGPLQARPLVALLDGRDCSIEMPILKDVATVAFCDAQSTSEIHEKVLNEAVGALMWHTIILTKEDLEKFKALRIIVRIGRIIVRI
AB011840 MDKNLMMPKRSRIDVKGNFANGPLQARPLVALLDGRDCSIEMPILKDVATVAFCDAQSTSEIHEKVLNEAVGALMWHTIILTKEDLEKFKALRIIVRIGRIIVRI
AY060646 GSGTDNIDVKAAGELGIAVCNVPGYGVEEVADTTMCLILNLYRRTYWLANMVREGKKFTGPEQVREAAHGCARIRGDTLGLVGLGRIGSAVALRAKAFGFNVIFY
CtBPL (Y.M.) GSGTDNIDVKAAGELGIAVCNVPGYGVEEVADTTMCLILNLYRRTYWLANMVREGKKFTGPEQVREAAHGCARIRGDTLGLVGLGRIGSAVALRAKAFGFNVIFY
AY069170 GSGTDNIDVKAAGELGIAVCNVPGYGVEEVADTTMCLILNLYRRTYWLANMVREGKKFTGPEQVREAAHGCARIRGDTLGLVGLGRIGSAVALRAKAFGFNVIFY
AB011840 GSGTDNIDVKAAGELGIAVCNVPGYGVEEVADTTMCLILNLYRRTYWLANMVREGKKFTGPEQVREAAHGCARIRGDTLGLVGLGRIGSAVALRAKAFGFNVIFY
AY060646 DPYLPDGIDKSLGLTRVYTLQDLLFQSDCVSLHCTLNEHNHHLINEFTIKQMRPGAFLVNTARGGLVDDETLALALKQGRIRAAALDVHENEPYNVFQGALKDAP
CtBPL (Y.M.) DPYLPDGIDKSLGLTRVYTLQDLLFQSDCVSLHCTLNEHNHHLINEFTIKQMRPGAFLVNTARGGLVDDETLALALKQGRIRAAALDVHENEPYNVFQGALKDAP
AY069170 DPYLPDGIDKSLGLTRVYTLQDLLFQSDCVSLHCTLNEHNHHLINEFTIKQMRPGAFLVNTARGGLVDDETLALALKQGRIRAAALDVHENEPYNVFQGALKDAP
AB011840 DPYLPDGIDKSLGLTRVYTLQDLLFQSDCVSLHCTLNEHNHHLINEFTIKQMRPGAFLVNTARGGLVDDETLALALKQGRIRAAALDVHENEPYN---GALKDAP
AY060646 NLICTPHAAFFSDASATELREMAATEIRRAIVGNIPDVLRNCVNKEYFMRTPPAAAAGGVAAAVYPEGLMGGYYTGALHHRAHSTTPHDGPHSTTMLGSTVGGGP
CtBPL (Y.M.) NLICTPHAAFFSDASATELREMAATEIRRAIVGNIPDVLRNCVNKEYFMRTPPAAAAGGVAAAVYPEG--------ALHHRAHSTTPHDGPHSTTMLGSTVGGGP
AY069170 NLICTPHAAFFSDASATELREMAATEIRRAIVGNIPDVLRNCVNKEYFMRTPPAAAAGGVAAAVYPEGKLQMISMQEK
AB011840 NLICTPHAAFFSDASATELREMAATEIRRAIVGNIPDVLRNCVNKEYFMRTPPAAAAGGVAAAVYPEGKLQMISMQEK
AY060646 TTVAQAAAAAVAAAAAAALLPSPVPSHLSPQVGGLPLGI-----PLSAPDPMMHLSSSIKTEVKAESTEAP
CtBPL (Y.M.) TTVAQAAAAAVAAAAAAALLPSPVPSHLSPQVGGLPLGIVSSQSPLSAPDPMMHLSSSIKTEVKAESTEAP

the splicing machinery favoring certain splice sites. $CtBP_L$ is generated from splicing originating within exon5 that joins core CtBP sequences (exons 1-5) to that of exon 6 and 7.

In this thesis, we study the expression and activities of two of these isoforms that we designate as $CtBP_L$ (479 aa) and $CtBP_S$ (383 aa) respectively. These two splice variants are found to mediate effective repression on reporter genes when assayed as Gal4 fusion proteins both in the cultured cells and in the embryo (Ryu and Arnosti, 2003; Sutrias-Grau and Arnosti, 2004). This prompts an understanding of whether CtBP isoforms in *Drosophila* are functionally equivalent or have distinct roles *in vivo*. Chordate genomes like those of human, mouse and *Ciona* are seen to contain only $CtBP_L$-like isoforms (not shown). $CtBP_S$ isoforms have only been described thus far in *Drosophila*, raising significant questions concerning the function of forms of this protein that do not contain the unstructured C-terminal extension.

REFERENCES

Arnosti, D. N. (2004). Multiple Mechanisms of Transcriptional Repression in Eukaryotes. In *Handbook of Experimental Pharmacology*, vol. 166 (ed. K. J. a. T. S. J. Gossen M.): Springer(Berlin).

Austin, R. J. and Biggin, M. D. (1995). A domain of the even-skipped protein represses transcription by preventing TFIID binding to a promoter: repression by cooperative blocking. *Mol Cell Biol* **15**, 4683-93.

Balasubramanian, P., Zhao, L. J. and Chinnadurai, G. (2003). Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. *FEBS Lett* **537**, 157-60.

Barnes, C. J., Vadlamudi, R. K., Mishra, S. K., Jacobson, R. H., Li, F. and Kumar, R. (2003). Functional inactivation of a transcriptional corepressor by a signaling kinase. *Nat Struct Biol* **10**, 622-8.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev* **16**, 1167-81.

Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev* **16**, 1964-76.

Bianchi-Frias, D., Orian, A., Delrow, J. J., Vazquez, J., Rosales-Nieves, A. E. and Parkhurst, S. M. (2004). Hairy transcriptional repression targets and cofactor recruitment in Drosophila. *PLoS Biol* **2**, E178.

Boyd, J. M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S. and Chinnadurai, G. (1993). A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *Embo J* **12**, 469-78.

Brannon, M., Brown, J. D., Bates, R., Kimelman, D. and Moon, R. T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development* **126**, 3159-70.

Cai, H. N., Arnosti, D. N. and Levine, M. (1996). Long-range repression in the Drosophila embryo. *Proc Natl Acad Sci U S A* **93**, 9309-14.

Chen, G. and Courey, A. J. (2000). Groucho/TLE family proteins and transcriptional repression. *Gene* **249**, 1-16.

Chen, G., Fernandez, J., Mische, S. and Courey, A. J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev* **13**, 2218-30.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol Cell* **9**, 213-24.

Chinnadurai, G. (2006). CtBP family proteins:unique transcriptional regulators in the nucleus with diverse cytosolic functions: Landes Bioscience.

Corda, D., Colanzi, A. and Luini, A. (2006). The multiple activities of CtBP/BARS proteins: the Golgi view. *Trends Cell Biol* **16**, 167-73.

Courey, A. J. and Jia, S. (2001). Transcriptional repression: the long and the short of it. *Genes Dev* **15**, 2786-96.

Criqui-Filipe, P., Ducret, C., Maira, S. M. and Wasylyk, B. (1999). Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation. *Embo J* **18**, 3392-403.

Deconinck, A. E., Mead, P. E., Tevosian, S. G., Crispino, J. D., Katz, S. G., Zon, L. I. and Orkin, S. H. (2000). FOG acts as a repressor of red blood cell development in Xenopus. *Development* **127**, 2031-40.

Deltour, S., Pinte, S., Guerardel, C., Wasylyk, B. and Leprince, D. (2002). The human candidate tumor suppressor gene HIC1 recruits CtBP through a degenerate GLDLSKK motif. *Mol Cell Biol* **22**, 4890-901.

Fang, M., Li, J., Blauwkamp, T., Bhambhani, C., Campbell, N. and Cadigan, K. M. (2006). C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. *Embo J* **25**, 2735-45.

Fjeld, C. C., Birdsong, W. T. and Goodman, R. H. (2003). Differential binding of NAD+ and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor. *Proc Natl Acad Sci U S A* **100**, 9202-7.

Furusawa, T., Moribe, H., Kondoh, H. and Higashi, Y. (1999). Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor deltaEF1. *Mol Cell Biol* **19**, 8581-90.

Gallop, J. L., Butler, P. J. and McMahon, H. T. (2005). Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission. *Nature* **438**, 675-8.

Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., Morrison, J. F., Ockuly, J., Stafstrom, C., Sutula, T. et al. (2006). 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat Neurosci.*

Gerasimova, T. I. and Corces, V. G. (2001). Chromatin insulators and boundaries: effects on transcription and nuclear organization. *Annu Rev Genet* **35**, 193-208.

Giot, L., Bader, J. S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y. L., Ooi, C. E., Godwin, B., Vitols, E. et al. (2003). A protein interaction map of Drosophila melanogaster. *Science* **302**, 1727-36.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr Opin Cell Biol* **8**, 358-64.

Grooteclaes, M., Deveraux, Q., Hildebrand, J., Zhang, Q., Goodman, R. H. and Frisch, S. M. (2003). C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs. *Proc Natl Acad Sci U S A* **100**, 4568-73.

Hamada, F. and Bienz, M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. *Dev Cell* **7**, 677-85.

Hasson, P., Muller, B., Basler, K. and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *Embo J* **20**, 5725-36.

Hidalgo Carcedo, C., Bonazzi, M., Spano, S., Turacchio, G., Colanzi, A., Luini, A. and Corda, D. (2004). Mitotic Golgi partitioning is driven by the membrane-fissioning protein CtBP3/BARS. *Science* **305**, 93-6.

Hildebrand, J. D. and Soriano, P. (2002). Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol* **22**, 5296-307.

Imai, S., Armstrong, C. M., Kaeberlein, M. and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795-800.

Katsanis, N. and Fisher, E. M. (1998). A novel C-terminal binding protein (CTBP2) is closely related to CTBP1, an adenovirus E1A-binding protein, and maps to human chromosome 21q21.3. *Genomics* **47**, 294-9.

Keller, S. A., Mao, Y., Struffi, P., Margulies, C., Yurk, C. E., Anderson, A. R., Amey, R. L., Moore, S., Ebels, J. M., Foley, K. et al. (2000). dCtBP-dependent and -independent repression activities of the Drosophila Knirps protein. *Mol Cell Biol* **20**, 7247-58.

Kim, J. H., Cho, E. J., Kim, S. T. and Youn, H. D. (2005). CtBP represses p300-mediated transcriptional activation by direct association with its bromodomain. *Nat Struct Mol Biol* **12**, 423-8.

Koipally, J. and Georgopoulos, K. (2000). Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity. *J Biol Chem* **275**, 19594-602.

Kumar, V., Carlson, J. E., Ohgi, K. A., Edwards, T. A., Rose, D. W., Escalante, C. R., Rosenfeld, M. G. and Aggarwal, A. K. (2002). Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell* **10**, 857-69.

Lawrence, P. A. (1992). The Making of a Fly: Blackwell Sci. Pub. (Oxford).

Levine, S. S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P. and Kingston, R. E. (2002). The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. *Mol Cell Biol* **22**, 6070-8.

Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D. and Lee, W. H. (1999). Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. *J Biol Chem* **274**, 11334-8.

Lin, X., Sun, B., Liang, M., Liang, Y. Y., Gast, A., Hildebrand, J., Brunicardi, F. C., Melchior, F. and Feng, X. H. (2003). Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Mol Cell* **11**, 1389-96.

Long, J., Zuo, D. and Park, M. (2005). Pc2-mediated sumoylation of Smad-interacting protein 1 attenuates transcriptional repression of E-cadherin. *J Biol Chem* **280**, 35477-89.

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G. et al. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* **298**, 1747-52.

Mannervik, M., Nibu, Y., Zhang, H. and Levine, M. (1999). Transcriptional coregulators in development. *Science* **284**, 606-9.

Melhuish, T. A. and Wotton, D. (2000). The interaction of the carboxyl terminus-binding protein with the Smad corepressor TGIF is disrupted by a holoprosencephaly mutation in TGIF. *J Biol Chem* **275**, 39762-6.

Meloni, A. R., Smith, E. J. and Nevins, J. R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. *Proc Natl Acad Sci U S A* **96**, 9574-9.

Molloy, D., Mapp, K. L., Webster, R., Gallimore, P. H. and Grand, R. J. (2006). Acetylation at a lysine residue adjacent to the CtBP binding motif within adenovirus 12 E1A causes structural disruption and limited reduction of CtBP binding. *Virology*.

Molloy, D. P., Barral, P. M., Bremner, K. H., Gallimore, P. H. and Grand, R. J. (2001). Structural determinants outside the PXDLS sequence affect the interaction of adenovirus E1A, C-terminal interacting protein and Drosophila repressors with C-terminal binding protein. *Biochim Biophys Acta* **1546**, 55-70.

Nardini, M., Spano, S., Cericola, C., Pesce, A., Massaro, A., Millo, E., Luini, A., Corda, D. and Bolognesi, M. (2003). CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. *Embo J* **22**, 3122-30.

Nardini, M., Svergun, D., Konarev, P. V., Spano, S., Fasano, M., Bracco, C., Pesce, A., Donadini, A., Cericola, C., Secundo, F. et al. (2006). The C-terminal domain of the transcriptional corepressor CtBP is intrinsically unstructured. *Protein Sci* **15**, 1042-50.

Nibu, Y., Senger, K. and Levine, M. (2003). CtBP-independent repression in the Drosophila embryo. *Mol Cell Biol* **23**, 3990-9.

Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S. and Levine, M. (1998a). dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the Drosophila embryo. *Embo J* **17**, 7009-20.

Nibu, Y., Zhang, H. and Levine, M. (1998b). Interaction of short-range repressors with Drosophila CtBP in the embryo. *Science* **280**, 101-4.

Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287**, 795-801.

Palmer, S., Brouillet, J. P., Kilbey, A., Fulton, R., Walker, M., Crossley, M. and Bartholomew, C. (2001). Evi-1 transforming and repressor activities are mediated by CtBP co-repressor proteins. *J Biol Chem* **276**, 25834-40.

Perdomo, J. and Crossley, M. (2002). The Ikaros family protein Eos associates with C-terminal-binding protein corepressors. *Eur J Biochem* **269**, 5885-92.

Phippen, T. M., Sweigart, A. L., Moniwa, M., Krumm, A., Davie, J. R. and Parkhurst, S. M. (2000). Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression. *J Biol Chem* **275**, 37628-37.

Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *Embo J* **17**, 2067-78.

Quinlan, K. G., Verger, A., Kwok, A., Lee, S. H., Perdomo, J., Nardini, M., Bolognesi, M. and Crossley, M. (2006). The role of the C-terminal binding protein PXDLS motif binding cleft in protein interactions and transcriptional repression. *Mol Cell Biol.*

Rutter, J., Reick, M., Wu, L. C. and McKnight, S. L. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**, 510-4.

Ryu, J. R. and Arnosti, D. N. (2003). Functional similarity of Knirps CtBP-dependent and CtBP-independent transcriptional repressor activities. *Nucleic Acids Res* **31**, 4654-62.

Schaeper, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T. and Chinnadurai, G. (1995). Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc Natl Acad Sci U S A* **92**, 10467-71.

Schmitz, F., Konigstorfer, A. and Sudhof, T. C. (2000). RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron* **28**, 857-72.

Senyuk, V., Sinha, K. K. and Nucifora, G. (2005). Corepressor CtBP1 interacts with and specifically inhibits CBP activity. *Arch Biochem Biophys* **441**, 168-73.

Sewalt, R. G., Gunster, M. J., van der Vlag, J., Satijn, D. P. and Otte, A. P. (1999). C-Terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. *Mol Cell Biol* **19**, 777-87.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A. and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941-53.

Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P., Nakatani, Y. and Shi, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**, 735-8.

Shi, Y. J., Matson, C., Lan, F., Iwase, S., Baba, T. and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. *Mol Cell* **19**, 857-64.

Sierra, J., Yoshida, T., Joazeiro, C. A. and Jones, K. A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev* **20**, 586-600.

Sollerbrant, K., Chinnadurai, G. and Svensson, C. (1996). The CtBP binding domain in the adenovirus E1A protein controls CR1-dependent transactivation. *Nucleic Acids Res* **24**, 2578-84.

Spano, S., Silletta, M. G., Colanzi, A., Alberti, S., Fiucci, G., Valente, C., Fusella, A., Salmona, M., Mironov, A., Luini, A. et al. (1999). Molecular cloning and functional characterization of brefeldin A-ADP-ribosylated substrate. A novel protein involved in the maintenance of the Golgi structure. *J Biol Chem* **274**, 17705-10.

Spyer, M. and Allday, M. J. (2006). The transcriptional co-repressor C-terminal binding protein (CtBP) associates with centrosomes during mitosis. *Cell Cycle* **5**, 530-7.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 201-19.

Strahl, B. D. and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* **403**, 41-5.

Struffi, P. and Arnosti, D. N. (2005). Functional interaction between the Drosophila knirps short range transcriptional repressor and RPD3 histone deacetylase. *J Biol Chem* **280**, 40757-65.

Struffi, P., Corado, M., Kulkarni, M. and Arnosti, D. N. (2004). Quantitative contributions of CtBP-dependent and -independent repression activities of Knirps. *Development* **131**, 2419-29.

Strunk, B., Struffi, P., Wright, K., Pabst, B., Thomas, J., Qin, L. and Arnosti, D. N. (2001). Role of CtBP in transcriptional repression by the Drosophila giant protein. *Dev Biol* **239**, 229-40.

Sundqvist, A., Sollerbrant, K. and Svensson, C. (1998). The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex. *FEBS Lett* **429**, 183-8.

Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol* **24**, 5953-66.

Turner, J. and Crossley, M. (2001). The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *Bioessays* **23**, 683-90.

Van Hateren, N., Shenton, T. and Borycki, A. G. (2006). Expression of avian C-terminal binding proteins (Ctbp1 and Ctbp2) during embryonic development. *Dev Dyn* **235**, 490-5.

van Vliet, J., Turner, J. and Crossley, M. (2000). Human Kruppel-like factor 8: a CACCC-box binding protein that associates with CtBP and represses transcription. *Nucleic Acids Res* **28**, 1955-62.

Verger, A., Quinlan, K. G., Crofts, L. A., Spano, S., Corda, D., Kable, E. P., Braet, F. and Crossley, M. (2006). Mechanisms directing the nuclear localization of the CtBP family proteins. *Mol Cell Biol* **26**, 4882-94.

Vo, N., Fjeld, C. and Goodman, R. H. (2001). Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP. *Mol Cell Biol* **21**, 6181-8.

Wang, S. Y., Iordanov, M. and Zhang, Q. (2006). c-Jun NH2-terminal kinase promotes apoptosis by down-regulating the transcriptional co-repressor CtBP. *J Biol Chem.*

Weigert, R., Silletta, M. G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E. V., Salmona, M. et al. (1999). CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature* **402**, 429-33.

Wray, G. A. (2003). Transcriptional regulation and the evolution of development. *Int J Dev Biol* **47**, 675-84.

Zhang, H. and Levine, M. (1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo. *Proc Natl Acad Sci U S A* **96**, 535-40.

Zhang, Q., Nottke, A. and Goodman, R. H. (2005). Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis. *Proc Natl Acad Sci U S A* **102**, 2802-7.

Zhang, Q., Piston, D. W. and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895-7.

Zhao, L. J., Subramanian, T., Zhou, Y. and Chinnadurai, G. (2006). Acetylation by p300 regulates nuclear localization and function of the transcriptional corepressor CtBP2. *J Biol Chem* **281**, 4183-9.

Zheng, L., Roeder, R. G. and Luo, Y. (2003). S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell* **114**, 255-66.

CHAPTER II

Developmental Expression and Phylogenetic Conservation of Alternatively Spliced Forms of the C-terminal Binding Protein Corepressor

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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 found 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.

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, 2003). 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, 2005). 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 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., 2002; 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). Similar to the expression pattern in mice, avian orthologs of *Ctbp1* and *Ctbp2* are expressed in overlapping regions and distinct domains suggesting that *Ctbp1* and *Ctbp2* might have unique roles in certain tissues (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). It is unknown whether the distinct developmental phenotypes seen with vertebrate CtBP mutants are largely a reflection of the genes' unique promoter activities, or whether differences in the proteins themselves play a large role.

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).

Drosophila CtBP is vital for embryonic development and embryos lacking dCtBP function exhibit grave defects in segmentation. 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: *Drosophila melanogaster* yw^{67} (Arnosti lab), *Drosophila sechellia, Drosophila mojavensis* (Tucson *Drosophila* Stock Center), *Drosophila virilis* (Dr. Scott Pitnick). *Tribolium castaneum* was a gift from Dr. Susan Brown (Kansas State University), *Anopheles gambiae* from Dr. Ned Walker (Michigan State University) and *Apis mellifera* from Dr. Zachary Huang (Michigan State University). 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 bovine serum albumin as the standard. The supernatant was mixed with Laemmli sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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′, $CtBP_S$ 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′. The transcript for tubulin was amplified using primers BWH 763 - 5′ CCGCCACCTTCATCGGCAAC 3′ and BWH 764 - 5′ TTAGTTCTCGTCGACCACAG 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 minutes 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 for CtBP transcripts and 25 cycles for the tubulin transcript. PCR products were visualized by agarose gel electrophoresis and quantitated using BioRad Quantity One software Version 4.4.1. . Three separate RNA preparations for

embryos and three for adults were quantitated and a scatter plot was generated in Excel. Linear fits to the data were used to determine relative amounts of $CtBP_L$ and $CtBP_S$ mRNA for each RNA preparation, and these amounts were compared to levels of total CtBP and to tubulin. Relative levels of $CtBP_S$ mRNA increase from embryo to adult by 3-8 fold, when normalized to either total CtBP or tubulin controls, whereas normalized $CtBP_L$ mRNA levels do not change significantly. The data shown in Figure II-1C is a representative result of RT-PCR analysis of biological triplicates that were each analyzed at least two times. In this particular dataset, $CtBP_S$ was upregulated 6 fold in adults when normalized to both total CtBP and tubulin controls.

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. Relative similarities were calculated based on the pairwise alignment with the *D. melanogaster* peptide sequence for exons 6+7 as a reference.

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 ($CtBP_L$) (Figure II-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).

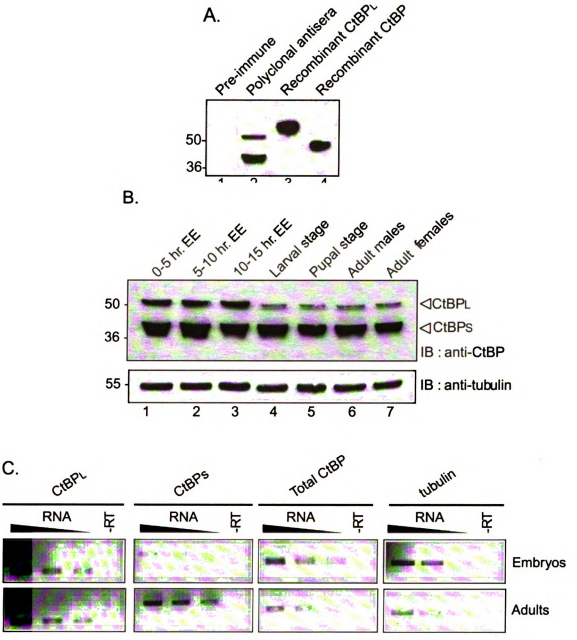
To analyze the developmental expression profile of CtBP isoforms, we analyzed soluble extracts from different developmental stages of the fly (Figure II-1B). Both $CtBP_L$ and $CtBP_S$ isoforms are detected throughout the first 15 hours of embryogenesis, with relatively higher levels of $CtBP_S$ than $CtBP_L$ (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 (Figure II-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).

Figure II-1: Developmental expression profile of CtBP isoforms in *Drosophila melanogaster*.

A. Specificity of anti-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 anti-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. $CtBP_S$ 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. Tubulin mRNA was measured as a control. 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. In this experiment, $CtBP_S$ levels increased from embryo to adult six-fold relative to either total CtBP or tubulin, while normalized $CtBP_L$ levels were unchanged.


A.
Pre-immune
Polyclonal antisera
Recombinant CtBPL
Recombinant CtBP
50
36
1
2
3
4
B.
0-5 hr. EE
5-10 hr. EE
10-15 hr. EE
Larval stage
Pupal stage
Adult males
Adult females
50
36
CtBPL
CtBPS
IB : anti-CtBP
55
IB : anti-tubulin
1
2
3
4
5
6
7
C.
CtBPL
CtBPs
Total CtBP
tubulin
RNA
-RT
Embryos
Adults

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 levels of $CtBP_S$ transcripts relative to total CtBP or tubulin mRNAs undergo a marked shift between these two stages, with $CtBP_S$ increasing 3-8 fold in the adults, while little change is seen in $CtBP_L$ levels (Figure II-1C). This change suggests that the protein profile favoring $CtBP_S$ later in development may be dictated 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

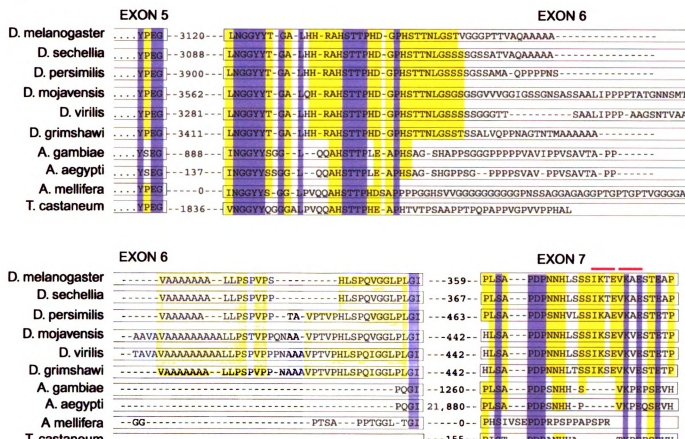
conserved CtBP sequences identified regions homologous to *D. melanogaster* exons 6 and 7, which encode the C-terminal extension of $CtBP_L$ (Figure II-2A). In *Drosophila* species, the sequences of exon 6 appear to be separated from an upstream exon by a ~3 kbp intron, while the intron is of smaller 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 (>92%), suggesting a lower level of evolutionary constraint (Figure II-2B). However, some motifs are conserved; 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 *T. castaneum*, suggesting that the downstream coding information is likely to be incorporated into mRNAs (Figure II-2C). 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 *T. castaneum* and *A. gambiae*, raising the possibility that acceptor sites in alternative locations may be used (Figure II-2C). In the bee, the open reading frame for the C-terminal extension is fused to the core sequences,

Figure II-2: Conservation of coding information for $CtBP_L$-specific C-terminus.

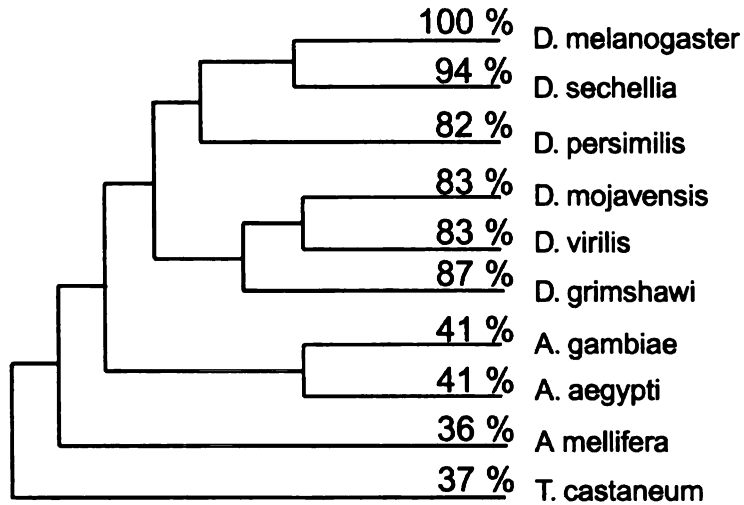
A. Peptide coding information present in dipterans, bee, and beetle genomic sequences homologous to alternatively spliced exon 6 and 7 in *Drosophila melanogaster* encoding CtBP "tail" region. Conceptual translations of genomic sequences are shown below sequence of $CtBP_L$, in which YPEG represents the end of the exon 5 coding sequence for the $CtBP_L$ isoform. Predicted intron size in nucleotides is indicated between exons. Introns in *Apis mellifera* have apparently been eliminated. Dark gray shading indicates widely conserved sequences; light gray shading partially conserved sequences. Possible sumoylation sites (I/V K X E) are indicated by gray 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 (not shown); this sequence is not conserved outside of *Drosophila*, unlike the residues 5′ of exon 6 shown in 2C.

B. Phylogenetic tree of species analyzed, displaying relative similarities of the C-terminal 'tail' in different species compared to *Drosophila melanogaster.* Pairwise comparisons were performed using *D. melanogaster* exons 6 and 7 (103 amino acids) as a reference. Percentage indicates identical amino acids or a conserved substitution. Residues present in expansions in more divergent species such as *D. mojavensis* and *D. virilis* were not scored. **C.** 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 this motif, thus alternative splicing may be a conserved feature here as well.

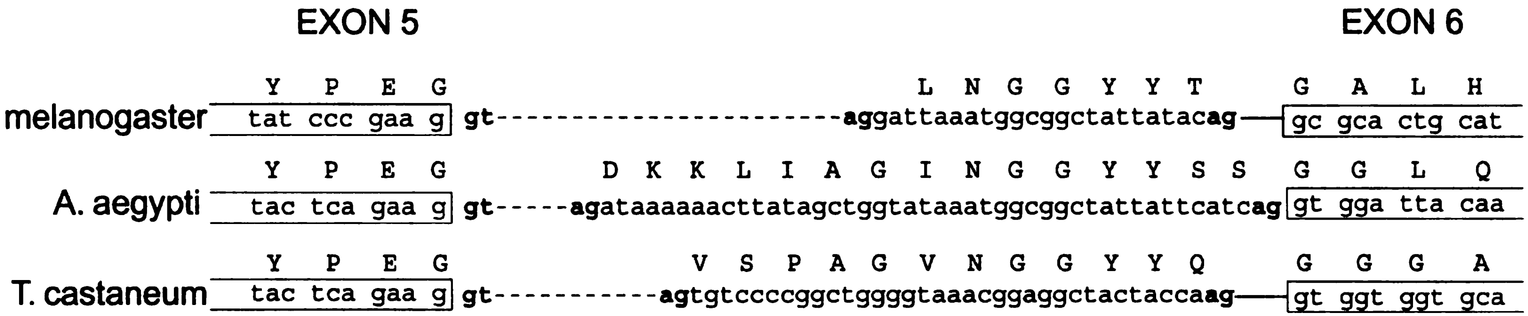
A.


EXON 5
EXON 6
D. melanogaster
D. sechellia
D. persimilis
D. mojavensis
D. virilis
D. grimshawi
A. gambiae
A. aegypti
A. mellifera
T. castaneum
EXON 6
EXON 7

B.


100 %
94 %
82 %
83 %
83 %
87 %
41 %
41 %
36 %
37 %
D. melanogaster
D. sechellia
D. persimilis
D. mojavensis
D. virilis
D. grimshawi
A. gambiae
A. aegypti
A mellifera
T. castaneum

C.


EXON 5
EXON 6
D. melanogaster
Y P E G
tat ccc gaa g
gt----------------------aggattaaatggcggctattatacag
L N G G Y Y T
G A L H
gc gca ctg cat
A. aegypti
Y P E G
tac tca gaa g
gt-----agataaaaaacttatagctggtataaatggcggctattattcatcag
D K K L I A G I N G G Y Y S S
G G L Q
gt gga tta caa
T. castaneum
Y P E G
tac tca gaa g
gt-----------agtgtccccggctggggtaaacggaggctactaccaag
V S P A G V N G G Y Y Q
G G G A
gt ggt ggt gca

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).

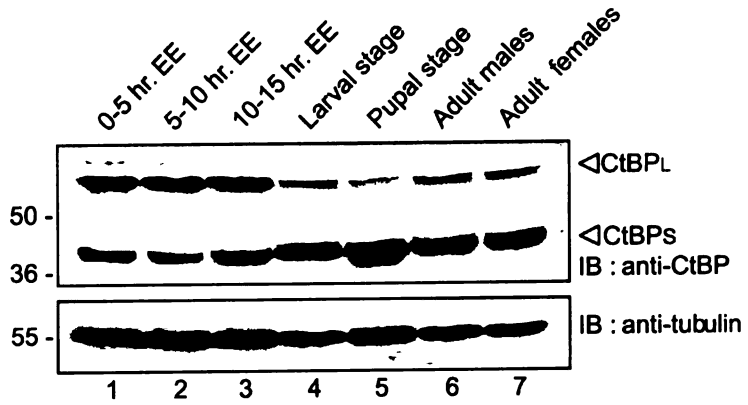
Developmental expression of alternative isoforms in *D. mojavensis* and *D. virilis*

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 (Figure II-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.

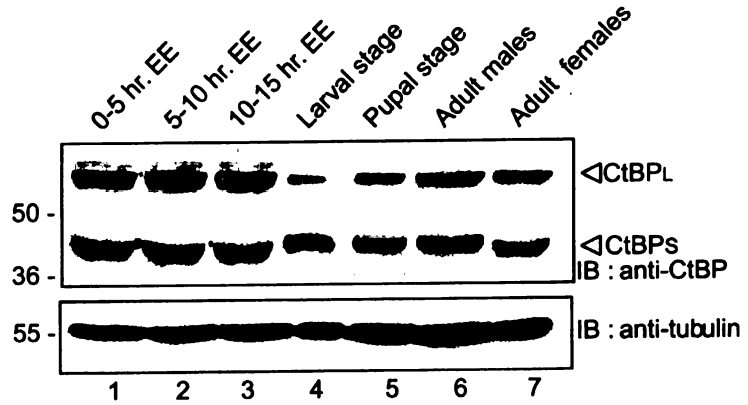
Figure II-3: Conserved developmental regulation of CtBP protein expression in *D. mojavensis* and *D. virilis*.

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. The bottom panels shows β-tubulin as a loading control.

A.


0-5 hr. EE
5-10 hr. EE
10-15 hr. EE
Larval stage
Pupal stage
Adult males
Adult females
◁CtBPL
◁CtBPS
IB : anti-CtBP
IB : anti-tubulin
50 -
36 -
55 -
1 2 3 4 5 6 7

B.


0-5 hr. EE
5-10 hr. EE
10-15 hr. EE
Larval stage
Pupal stage
Adult males
Adult females
◁CtBPL
◁CtBPS
IB : anti-CtBP
IB : anti-tubulin
50 -
36 -
55 -
1 2 3 4 5 6 7

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 (Figure II-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) (Figure II-4). Relative to *D. melanogaster*, the closely related *D. sechellia*, (diverged ~3 Mya) expresses $CtBP_S$ 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 honey bee, 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 *T. castaneum*, similar in mobility to *D. melanogaster* $CtBP_L$, 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

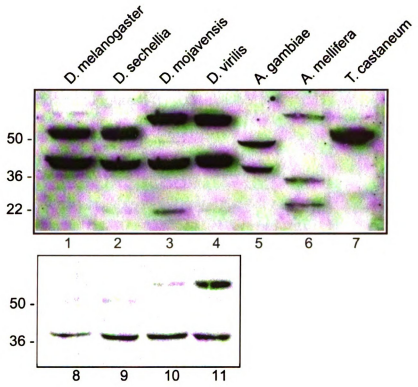


Figure II-4: Adult expression of CtBP proteins in four *Drosophila* species, *Anopheles gambiae, Apis mellifera*, and *Tribolium castaneum*.

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 *T. castaneum*. 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.

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* (Figure II-4, lanes 8-11), which is consistent with the developmental profiles for *D. virilis* and *D. mojavensis* shown in Figure II-3.

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 by the Pak1 kinase, 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. Our measurements of relative levels of CtBP mRNAs in embryos and adults indicate that an increase in the amount of the $CtBP_S$ spliceform may account for the shift toward this form of the protein later in the adult. However, differences in mRNA translation efficiency or stability of the two forms of the protein may also play a role. Phosphorylation of vertebrate CtBP by HIPK2 and c-Jun amino-terminal kinases at ser 422 in the unstructured C-terminal portion of the protein triggers degradation of the protein via the proteasome (Zhang et al., 2005; Wang et al., 2006). It is possible that a similar modification may account for the drop in *Drosophila* $CtBP_L$ levels.

We note that putative sumoylation signals are conserved in Dipteran sequences (Figure II-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.

ACKNOWLEDGMENTS

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REFERENCES

Balasubramanian P, Zhao LJ, Chinnadurai G (2003) Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. FEBS Lett **537**: 157-160

Barnes CJ, Vadlamudi RK, Mishra SK, Jacobson RH, Li F, Kumar R (2003) Functional inactivation of a transcriptional corepressor by a signaling kinase. Nat Struct Biol **10**: 622-628

Chinnadurai G (2003) CtBP family proteins: more than transcriptional corepressors. BioEssays **1**: 9-12

Chinnadurai G (2005) CtBP Family Proteins: Unique Transcriptional Regulators in the Nucleus with Diverse Cytosolic Functions. In: G. Chinnadurai (ed) CtBP Family Proteins. Eurekah.com, Landes Bioscience, Georgetown, Texas, pp 1-17

Corda D, Colanzi A, Luini A (2006) The multiple activities of CtBP/BARS proteins: the Golgi view. Trends Cell Biol **16**: 167-173

Fang M, Li J, Blauwkamp T, Bhambhani C, Campbell N, Cadigan KM (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in *Drosophila*. EMBO J **25**: 2735-2745

Gallop JL, Butler PJ, McMahon HT (2005) Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission. Nature **438**: 675-678

Hildebrand JD, Soriano P (2002) Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. Mol Cell Biol **15**: 5296-5307

Kagey MH, Melhuish TA Wotton D (2003) The polycomb protein Pc2 is a SUMO E3. Cell **1**: 127-137

Kumar V, Carlson JE, Ohgi KA, Edwards TA, Rose DW, Escalante CR, Rosenfeld MG Aggarwal AK (2002) Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. Mol Cell **4**: 857-869

Li X, Noll M. (1994) Compatibility between enhancers and promoters determines the transcriptional specificity of gooseberry and gooseberry neuro in the *Drosophila* embryo. EMBO J **13**: 400-406

Lin X, Sun B, Liang M, Liang YY, Gast A, Hildebrand J, Brunicardi FC, Melchior F Feng XH (2003) Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. Mol Cell **5**: 1389-1396

Linding R, Russell RB, Neduva V, Gibson TJ (2003) GlobPlot: Exploring

protein sequences for globularity and disorder. Nucleic Acids Res **31**: 3701-3708

Nardini M, Spanò S, Cericola C, Pesce A, Massaro A, Millo E, Luini A, Corda D, Bolognesi M (2003) CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. EMBO J **22**: 3122 – 3130

Nardini M, Svergun D, Konarev PV, Spano S, Fasano M, Bracco C, Pesce A, **Donadini A, Cericola C, Secundo F, Luini A, Corda D Bolognesi M** (2006) The C-terminal domain of the transcriptional corepressor CtBP is intrinsically unstructured. Protein Sci **15**: 1042-1050

Nibu Y, Zhang H, Bajor E, Barolo S, Small S, Levine M (1998a) dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the *Drosophila* embryo. EMBO J **23**: 7009-7020

Nibu Y, Zhang H, Levine M (1998b) Interaction of short-range repressors with *Drosophila* CtBP in the embryo. Science **5360**: 101-104

Poortinga G, Watanabe M, Parkhurst SM (1998) *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. EMBO J **7**: 2067-2078

Riefler GM, Firestein BL (2001) Binding of neuronal nitric-oxide synthase (nNOS) to carboxyl-terminal- binding protein (CtBP) changes the localization of CtBP from the nucleus to the cytosol: a novel function for targeting by the PDZ domain of nNOS. J Biol Chem **51**: 48262-48268

Schmitz F, Konigstorfer A, Sudhof TC (2000) RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. Neuron **28**: 857-872

Struffi P, Arnosti DN (2005) Functional interaction between the *Drosophila* knirps short range transcriptional repressor and RPD3 histone deacetylase. J Biol Chem **280**: 40757-40765

Sutrias-Grau M, Arnosti DN (2004) CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. Mol Cell Biol **24**: 5953-5966

Turner J, Crossley M (2001) The CtBP family: enigmatic and enzymatic transcriptional co-repressors. BioEssays **8**: 683-690

Van Hateren N, Shenton T, Borycki AG (2006) Expression of avian C-terminal binding proteins (Ctbp1 and Ctbp2) during embryonic development. Dev Dyn **235**: 490-495

Wang SY, Iordanov M, Zhang Q (2006) c-Jun-NH2-terminal kinase promotes apoptosis by down-regulating the transcriptional corepressor CtBP. J Biol

Chem [in press, Epub ahead of print]

Weigert R, Silletta MG, Spano S, Turacchio G, Cericola C, Colanzi A, Senatore S, Mancini R, Polishchuk EV, Salmona M, Facchiano F, Burger KN, Mironov A, Luini A, Corda D (1999) CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. Nature **6760**: 429-433

Zhang Q, Nottke A, Goodman RH (2005) Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis. Proc Natl Acad Sci U S A **102**: 2802-2807

Zhang Q, Piston DW, Goodman RH (2002) Regulation of corepressor function by nuclear NADH. Science **5561**: 1895-1897

Zhao LJ, Subramanian T, Zhou Y, Chinnadurai G (2006) Acetylation by p300 regulates nuclear localization and function of the transcriptional corepressor CtBP2. J Biol Chem **281**: 4183-4189

CHAPTER III

FUNCTIONAL ANALYSIS OF CtBP DEHYDROGENASE-LIKE DOMAIN

ABSTRACT

The transcriptional corepressor CtBP lies at the center of several protein networks involved in gene expression, development and oncogenesis. In this study, we have sought to uncover the functional relevance of the dehydrogenase-like domain for CtBP biological function in a whole organism context. Our observations show that *Drosophila* CtBP requires NAD binding for biological activity *in vivo*. NAD binding by CtBP has been postulated to allow formation of higher order complexes by CtBP. Our results show that dimerization of CtBP proteins is not dependent upon NAD. However, the binding of NAD might be critical for inducing allosteric changes in CtBP that perhaps allow for subsequent recruitment of cofactors needed for transcriptional repression.

Introduction

A hallmark of *Drosophila* development is the regulation of gene expression by transcriptional repression (Gray and Levine, 1996). Multiple mechanisms effecting repression have been identified that have increased our understanding of gene regulation, but the characterization of how and when these mechanisms are utilized is still in its rudimentary stages. A general functional distinction has been made categorizing the range of repressor action in *Drosophila* (Gray and Levine, 1996). Short-range repressors are locally-acting proteins that are fundamental regulators of gene expression and segmentation in *Drosophila*. Repression by these proteins is possible when they are located within a short distance (~100-150 bp) of either activators or the transcription initiation site, and their short range of action means that individual enhancers can be repressed without interfering with the functioning of other nearby enhancers.

All short-range repressors studied in *Drosophila* employ the C-terminal Binding Protein (CtBP) as an effector to mediate repression. CtBP was first identified in human cells through its interaction with the adenoviral E1A oncoprotein where it was found to repress E1A oncogenicity (Boyd et al., 1993). Since then, numerous transcription factors have been found to employ this conserved corepressor (Chinnadurai, 2002). Several vertebrate and invertebrate DNA-binding proteins are known to employ CtBP for their biological functions (Criqui-Filipe et al., 1999; Furusawa et al., 1999; Kegel et al., 2002; Koipally and Georgopoulos, 2000). Mice mutant for *CtBP1* and *CtBP2* genes display grave developmental defects that may be ascribed to

the compromised abilities of numerous regulators of gene expression, emphasizing the importance of CtBP in development (Hildebrand and Soriano, 2002). The *Drosophila* homolog of CtBP contributes quantitatively to total repressor activity to generate appropriate levels of repression by short-range repressors like Knirps and Krüppel (Keller et al., 2000; Struffi et al., 2004; Sutrias-Grau and Arnosti, 2004). CtBP also interacts with the long-range repressor Hairy; in this case, however, CtBP appears to antagonize rather than facilitate repression by Hairy's primary cofactor Groucho, through a competitive binding mechanism (Zhang and Levine, 1999).

Multiple mechanisms may be used by transcriptional repressors to enhance the overall repression output or to provide unique activities that regulate transcription negatively at a given promoter (Arnosti, 2004). Recent work has established that the repression activity of CtBP proteins relies on the formation of a multiprotein assembly containing an assortment of enzymes. This might represent one mechanism by which CtBP acts to effect transcriptional repression, namely as an adaptor that links chromatin-modifying activities to target promoters. The CtBP1 corepressor complex purified from human cells includes chromatin remodeling enzymes that mediate histone modifications by deacetylation and methylation of H3-K9, and the demethylation of H3-K4 (Shi et al., 2003). An alternate mechanism posits that CtBP directly binds bromodomains of histone acetyltransferases such as p300 and CBP, and blocks them from contacting and modifying histone tails (Kim et al., 2005; Senyuk et al., 2005).

Growing evidence indicates that CtBP proteins are multifunctional performing roles in addition to controlling gene expression. Multiple CtBP

isoforms have been implicated in cytosolic functions such as Golgi maintenance and synaptic ribbon formation in retinal cells (Chinnadurai, 2006b; Corda et al., 2006; Schmitz et al., 2000).

Despite the multiplicity of CtBP isoforms, all CtBP proteins exhibit a highly conserved signature sequence and structural similarity to alpha hydroxy acid dehydrogenases (Chinnadurai, 2002; Kumar et al., 2002). This similarity extends over a centrally located NAD+/NADH dinucleotide-binding domain and a triad of residues known to be important for mediating catalysis in related dehydrogenase enzymes. The discovery of an NAD-binding fold within CtBP has provoked several studies that attempt to put CtBP at the focus of redox-regulated pathways. Regulation of gene expression by redox regulators represents an emerging concept in transcription (Imai et al., 2000; Rutter et al., 2001).

In addition to possibly serving in a catalytic mode, NAD is also suggested to play a role in modulation of CtBP structure. NAD binding to CtBP has been shown to promote oligomerization of the protein in vitro (Balasubramanian et al., 2003). Possibly as a consequence of this, mutations that disrupt NAD binding affect CtBP activity. Mutations that impede NAD binding (G181A, G183A and D204A) reduce CtBP1 binding to an E1A-GST fusion protein and prevent CtBP mediated repression of a Gal4-E1A activated reporter in cell culture (Kumar et al., 2002). An NAD binding mutant of CtBP2 (G189A) de-represses a heterologous SV40 enhancer-activated reporter in C33 cells (Thio et al., 2004). In one case, a CtBP protein mutated in the NAD binding cleft was found to retain activity (Grooteclaes et al., 2003). One problem with these assays is that the transiently transfected reporters do not

represent a physiological chromatin environment. Thus, it is not certain whether relevant CtBP chromatin modifying activities are being assayed. In contrast, whole animal assays using integrated reporter genes test gene activity in a native chromatin context. We have assayed CtBP proteins in such a setting and found that mutations in key glycines (G181A, G183A) implicated in NAD binding abolish repression by Gal4-CtBP fusion proteins (Sutrias-Grau and Arnosti, 2004). This result suggests that NAD binding is crucial in this context.

α-hydroxy acid dehydrogenases contain a well-conserved triad of active site residues. This catalytic center of related dehydrogenases is well conserved in vertebrate and invertebrate CtBP family members. Triple mutations that disrupt the putative catalytic ability (H315Q, G295, R266) were seen to diminish CtBP1 interaction with an E1A-GST chimera, and reduced repression of a Gal4-E1A activated reporter in cell culture (Kumar et al., 2002). These mutations were also seen to abrogate a weak dehydrogenase activity seen with the wild type vertebrate CtBP1 protein. In contrast, a single mutation disrupting the putative active site (H315Q) did not obstruct interaction with E1A and this mutant was found to retain repressive potential comparable to the wild type CtBP protein (Grooteclaes et al., 2003). In whole animal assays using integrated reporters, mutations in the putative active site of CtBP abolished repression (Sutrias-Grau and Arnosti, 2004). This result suggests that the presumptive catalytic function is not required for CtBP mediated transcriptional repression.

MATERIALS AND METHODS

Construction of P-element vectors

cDNA fragments for CtBP isoforms (Accession numbers: AY060646 for $dCtBP_L$ and AB011840 for $dCtBP_S$) carrying a C-terminal double FLAG tag (FF) were introduced downstream of 5X UAS sites in the pUAST vector using unique KpnI-XbaI sites in the polylinker (Brand and Perrimon, 1993). An optimal Kozak sequence and start codon was provided by the insertion of an adaptor upstream of the cDNA sequence using BglII and KpnI restrictions sites (DA 683 5'-GATCACCCGGGACCAAAATGGGTAC-3' and DA 684 5'-CCATTTTGGTCCCGGGT-3'). $dCtBP_L$ NAD binding and catalytic mutants have been described elsewhere and contain mutations in G181A, G183A disrupting NAD binding (NAD) and H315Q abolishing the putative active site (CAT) (Sutrias-Grau and Arnosti, 2004). For the dimerization mutant, point mutations were introduced in $dCtBP_L$-FF at four key arginines (R141A, R142A, R163A and R171A). All mutants described were made in the context of the full length $CtBP_L$. The constructs expressing CtBP ubiquitously were generated in the pUAST vector containing CtBP wild type and mutant cDNA fragements. The vector has a basal hsp70 promoter and 5X UAS sites that were removed by restriction digestion with SphI and KpnI and replaced with an adaptor that containing SphI, a new AscI and KpnI restriction sites and an optimal Kozak sequence (DA 885 5'-CACCGGCGCGCCACCAAAATGGGTAC-3'; DA 886 5'-CCATTTTGGTGGCGCGCCGGTGCATG-3'). The promoter region of CtBP

(~8054 bp) was amplified from embryo genomic DNA in two pieces: the first 4000 bp fragment containing one of the predicted start sites was amplified as an SphI-AscI fragment (CG8841302-8845092) with DA 926 (5'-GTGCATGCGAAATGGTTAGCCAGCGTGGTG-3') and DA 927 (5'-CGGGCGCGCCTTGAAATCGAGAATCCTGCAATGG-3') and inserted immediately upstream of the cDNA in frame. The second fragment (~4054 bp) with the other putative transcriptional start site was amplified as an SphI-SphI fragment (CG8837323-8841301) with primer sets DA 924 (5'-CTGCATGCATACCATAATTCTTGCAGTTTGCC-3') and DA 925 (5'-CGGCATGCAGCTTTCTGTTTCATGCATATGCAC -3') and introduced into the SphI site, upstream of the first fragment (See Appendix A, Figure A-1).

Immunoprecipitations and Western Blot Analysis

0-12 hour embryos were collected from transgenic lines expressing FLAG-tagged CtBP proteins, resuspended in lysis buffer (150mM NaCl, 50 mM HEPES, pH 7.9, 10% glycerol, 0.1mM EDTA with Complete mini-EDTA free protease inhibitor cocktail, Roche) and sonicated using a Branson-250 sonifier. Soluble lysates (~20 mgs total protein) were immunoprecipitated overnight at 4°C with 25 µl of anti-M2 (Sigma) crosslinked to protein G beads, by incubation with the chemical crosslinker dimethyl pimelimidate dihydrochloride to a final concentration of 20mM. Immunoprecipitation with mouse monoclonal IgG and anti-M2 with non-transgenic embryo extracts was performed in parallel as negative controls. Beads were collected and washed thrice in lysis buffer and eluted in 40 µl of Laemmli sample buffer.

Immunoprecipitates were separated on SDS-PAGE gels and analyzed by western blotting as described below using rabbit polyclonal anti-CtBP (1:10,000) (Struffi and Arnosti, 2005).

Total extracts from adult flies were immunoblotted 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. Mouse monoclonal antibodies include anti-M2 used to detect FLAG epitope-tagged CtBP (1:10,000) and tubulin (1:6000, Iowa Hybridoma Bank). These were visualized using HRP-conjugated secondary antibodies (Pierce) and SuperSignal® West Pico chemiluminiscent substrate (Pierce).

P-element transformation and antibody staining of imaginal discs

All the P-element transformation vectors described were introduced into *Drosophila* germ line by injection of yw^{67} embryos as described previously.

Imaginal discs dissected from third instar larvae were fixed in antibody staining buffer (10 mM potassium phosphate, pH 6.8, 15 mM NaCl, 45 mM KCl and 2 mM MgCl2) and 4% formaldehyde on ice for 45 minutes. For detection of FLAG-tagged proteins, anti-M2 was used at a concentration of 1:1500. This antibody was detected and developed using a universal secondary antibody conjugated to HRP (1:250, Vectastain ABC Kit). Discs were mounted in 80% glycerol and the expression pattern was visualized using an Elite PK-62000 Universal Vectastain ABC kit (Vector Laboratories,

Inc., Manufacturer's protocol).

Recombinant CtBP proteins and Gel filtration chromatography

The cDNAs for wild type CtBP (both $CtBP_L$ and $CtBP_S$) and the three mutants (NAD binding mutant, catalytic mutant and dimerization defective mutant of CtBP) were cloned into a modified pET-15b expression vector using Kpn I and Not I restriction sites (created by D.N. Arnosti). This vector provides an N-terminally fused hexahistidine tag. Except for the catalytic mutant cDNA that contains 2 FLAG-tags at the C-terminus, all other cDNAs were designed to carry a single tag. These vectors were then used to transform heat shock competent *E.coli* strain BL21 (DE3). 25 ml of log phase bacterial cultures were induced with 0.8mM isopropyl thiogalactose (IPTG) overnight at 16°C. The cells were collected, resuspended in buffer containing 50mM HEPES pH 7.9, 150mM NaCl, 10% glycerol, 10 mM imidazole with a Roche cocktail protease inhibitor tablet and 10 mM β-mercaptoethanol, sonicated and centrifuged to separate the soluble supernatant fraction. 0.1% Triton X was added to supernatant before incubation with Ni^{2+}-NTA beads (Sigma) at 4°C for 3 hours. The beads were washed thrice in the same buffer with 20 mM imidazole and eluted with 250 mM imidazole.

~10 µg of purified protein was fractionated on a Superdex 200 10/30 size exclusion column (Amersham) equilibrated with buffer containing 150 mM NaCl, 50mM HEPES pH 7.9, 0.1 mM EDTA and 10% glycerol. Following sample injection, 0.5 ml fractions were collected at a flow rate of 0.5 ml/min. Pools of three fractions (4 µl each fraction) were resolved on SDS-PAGE gels

and analyzed by western blotting using anti-M2 to detect FLAG-tagged recombinant proteins as described before (1:10,000). For the $CtBP_L$-NAD mutant a pool of three fractions (10 µl each fraction) was analyzed by western blots. Size markers (MW-GF-1000, Sigma) were separated under similar conditions.

RESULTS

Assaying the biological activity of wild type and mutant CtBP proteins *in vivo*

We have previously observed that an NAD binding mutant of CtBP (G181A, G183A) is impaired for repression, whereas a putative catalytic site mutant (H315Q) retains its repression activity when tested as Gal4 tethered proteins in embryo repression assays, suggesting that NAD binding is required for CtBP repression activity (Sutrias-Grau and Arnosti, 2004). However, these assays are limited in that they only test the protein's activity in a repression context, and as a DNA binding fusion protein. To investigate the functional relevance of residues within the dehydrogenase-like domain of CtBP in a more general context, we generated *Drosophila* transgenic lines expressing proteins that carry mutations in conserved residues. The changes introduced are predicted to disrupt NAD binding (NAD), disrupt the putative catalytic site (CAT) or abrogate dimerization (DIM) based on the effects of these mutations in the highly similar vertebrate CtBP1 (Balasubramanian et al., 2003; Kumar et al., 2002; Nardini et al., 2003). NAD binding by CtBP has been suggested to promote oligomerization that might lead to recruitment of cofactors effecting repression on a CtBP-dependent target (Balasubramanian et al., 2003).

We overexpressed the wild type CtBPL and the three mutant proteins using the UAS-Gal4 system (For UAS-Gal4 assay see Appendix B - Figure B-1). Ectopic expression of the wild type protein and a putative catalytic mutant

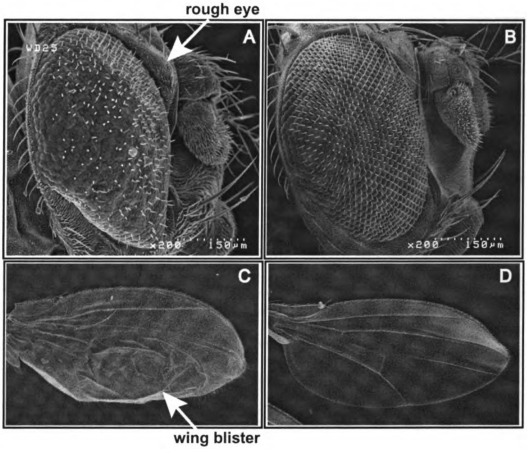


Figure III-1: Phenotypes induced by overexpression of wild type and mutant CtBP proteins

Misexpression of CtBP proteins in eye (**A**) induces rough eye phenotype, and in wing (**C**) induces vein abnormalities and blistering. Normal eye and wing appearance shown in **B** and **D**. Phenotypes were observed with misexpressed wild type $CtBP_L$ and $CtBP_L$ (CAT). Misexpression of $CtBP_L$ (NAD) and $CtBP_L$ (DIM) mutants and the short splice variant of CtBP did not yield any abnormal phenotype. Expression was achieved with eye specific *GMR-Gal4* driver or the wing-specific *en-Gal4* driver.
For a schematic of UAS-Gal4 assay, see Appendix B-Figure B-1.
A minimum of three independent lines per transgene were examined for phenotypic outcomes. SEM images are courtesy of Geoffrey Williams, Brown University.

Table III-1: Quantitation of CtBP misexpression phenotypes

Flies with phenotypes generated by wild-type CtBP isoforms and mutant forms that affect the $CtBP_L$ (CAT), $CtBP_L$ (NAD), or $CtBP_L$ (DIM) residues were scored. $CtBP_L$ and $CtBP_L$ (CAT) mutants showed similar effects; no wing or eye phenotypes were observed with the $CtBP_L$ (NAD and $CtBP_L$ (DIM) mutants or the $CtBP_S$ isoform. A minimum of three independent lines per transgene were counted for phenotypes.

	EYE PHENOTYPES			WING PHENOTYPES		
Transgene	Rough eyes(%)	# lines tested	n=	Wing defects(%)	# lines tested	n=
CtBPL w.t.	79-87	4	758	8-30	4	490
CtBPL CAT-	84-91	3	272	20-42	4	958
CtBPL NAD-.	0	3	580	0	3	724
CtBPL DIM-	0	6	618	0	4	436
CtBPs w.t.	0	3	272	0	3	370
none	0	-	210	0	-	124

of CtBP (H315Q mutation) induced wing blisters and rough eyes when misexpressed in the wing using an *en-Gal4* driver and in the eye using the *GMR-Gal4* driver. In contrast, misexpression of the NAD binding mutant, dimerization mutant, or the short isoform of CtBP induced no obvious phenotype in this assay (Figure III-1, Table III-1).

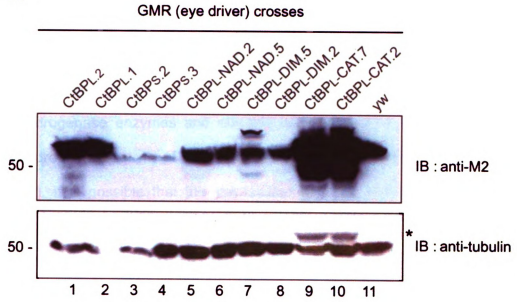
To test if the differences in induced phenotypes reflected intrinsic differences in protein activity or rather in levels of protein expression, adult flies were assayed for CtBP protein levels by immunoblotting and larvae were assayed by immunostaining of eye imaginal discs. The CtBP proteins carry a double FLAG-tag at the C-terminus that permits detection of the recombinant protein by an anti-M2 antibody. As shown in Figure III-2, the steady state protein levels $CtBP_L$ (NAD), $CtBP_L$ (DIM) and $CtBP_S$ were significantly lower than the wild type $CtBP_L$ or $CtBP_L$ (CAT). The low expression of $CtBP_L$ (DIM) suggests that lack of dimerization may destabilize the protein, which raises the possibility that the $CtBP_L$ (NAD) mutant protein may also be poorly expressed due to lack of dimerization. The expression of $CtBP_L$ (CAT) protein was consistently observed to be comparable to the wild type protein for several independent lines tested.

Figure III-2: Relative steady state levels of recombinant CtBP proteins: $CtBP_L$ (NAD) and $CtBP_L$ (DIM) mutants and $CtBP_S$ isoform are expressed at low levels.

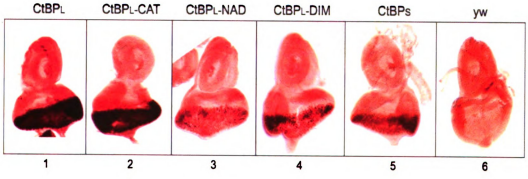
A. Single adult flies misexpressing the transgene were solubilized in Laemmli sample buffer and extracts were probed with anti-M2 antibody to detect the recombinant FLAG-tagged proteins. The $CtBP_L$ (NAD) (lanes 5,6), or $CtBP_L$ (DIM) (lanes 7,8) were detected at low levels compared to $CtBP_L$ (lanes 1,2) and $CtBP_L$ (CAT) (lanes 9,10). $CtBP_S$ isoform was practically undetectable, although upon darker exposure a faint band could be observed (lanes 3, 4; data not shown). The lower panel shows the same blot stripped and reprobed with β-tubulin antibody as a loading control. β-tubulin was reduced in lanes 1-3, indicating these lanes may be underloaded. Two independent lines per transgene are shown.

B. Third instar wing imaginal discs were dissected from third instar larval progeny misexpressing the transgenes described above. Discs were immunostained with anti-M2 (1:1500). The eye driver *GMR-Gal4* expresses CtBP in the posterior half of the imaginal disc. $CtBP_L$ and $CtBP_L$ (CAT) showed comparable expression (Panels 1, 2) while the $CtBP_L$ (NAD) (Panel 3), $CtBP_L$ (DIM) (Panel 4) and $CtBP_S$ (Panel 5) were expressed at low levels. Discs from *GMR-Gal4* crossed to non-transgenic flies served as negative control (Panel 6).

A.


GMR (eye driver) crosses
CtBPL.2
CtBPL.1
CtBPS.2
CtBPS.3
CtBPL-NAD.2
CtBPL-NAD.5
CtBPL-DIM.5
CtBPL-DIM.2
CtBPL-CAT.7
CtBPL-CAT.2
yw
50 -
IB : anti-M2
50 -
*
IB : anti-tubulin
1 2 3 4 5 6 7 8 9 10 11

B.


CtBPL
CtBPL-CAT
CtBPL-NAD
CtBPL-DIM
CtBPs
yw
1
2
3
4
5
6

Drosophila $CtBP_L$ presumptive catalytic mutant is competent for dimerization

Consistent with previous cell-based and embryo repression assays, mutation of the well-conserved histidine residue does not measurably affect CtBP biological activity. The mutated histidine is important for catalysis in dehydrogenase enzymes and reported to be critical for the weak in vitro dehydrogenase activity of $CtBP_L$ (Balasubramanian et al., 2003; Kumar et al., 2002). It is possible that this presumptive catalytic residue in CtBP might function in a different biological context not tested in our assay. In addition, the mutant protein may form heterodimers, recruiting one wild-type CtBP that may provide enough function to mask the effect of the mutation (Figure III-3).

To test this possibility, co-immunoprecipitations were performed on embryo extracts expressing recombinant, epitope-tagged mutants ubiquitously. Immunoprecipitation using the anti-M2 FLAG antibody recovered the epitope tagged $CtBP_L$ (CAT) protein, as well as endogenous $CtBP_L$ and $CtBP_S$ proteins (Figure III-3). The association of $CtBP_L$ (CAT) with endogenous CtBP proteins sheds new light on our previous embryo repression assays in which Gal4-$CtBP_L$ (CAT) was assayed (Sutrias-Grau and Arnosti, 2004). This result suggests that the repression activity of the catalytic mutant might be attributable to its dimerization with wild-type endogenous proteins. To better characterize the impact of this specific mutation, a CtBP null background is desired where possible heterodimerization can be ruled out (See Appendix A).

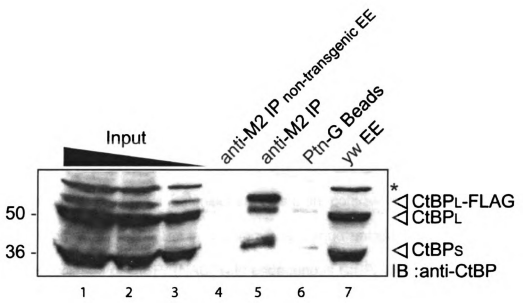


Figure III-3: $CtBP_L$ (CAT) protein is capable of dimerization

Embryo extracts prepared from transgenic lines that express recombinant $CtBP_L$ (CAT) (FLAG-tagged) from CtBP promoter, were immunoprecipitated with anti-M2 (lane 5). Endogenous CtBP isoforms specifically co-precipitated with the recombinant protein, and no non-specific associations were observed with either beads alone (lane 6) or anti-M2 IP of non transgenic embryo extracts (lane 4). A sample of non-transgenic embryo extract (yw EE) was loaded as a size marker to judge migration of CtBP proteins (lane 7). A non-specific band observed is marked with an asterisk.

Drosophila $CtBP_L$ and $CtBP_S$ can homo- and heterodimerize

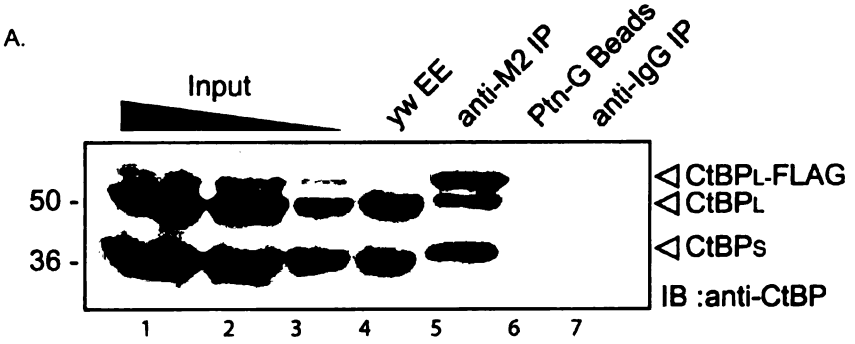
The ability of CtBP to dimerize is predicted to be central to its role as a bridging factor, allowing one monomeric unit to contact a DNA bound factor and the other to interact with chromatin modifying factors. Vertebrate CtBP proteins are known to dimerize, and dimerization may allow the formation of a CtBP repressor assembly. All CtBP isoforms identified in vertebrates possess unstructured C-terminal extensions ('tails'), in contrast to *Drosophila* that expresses a tailed ($CtBP_L$) in addition to a unique untailed isoform ($CtBP_S$). The association of $CtBP_L$ (NAD) with endogenous $CtBP_L$ and $CtBP_S$ suggests that hetero and homodimers between tailed and untailed proteins are able to form. To test if wild type $CtBP_L$ and $CtBP_S$ proteins can dimerize, we performed co-immunoprecipitations to test the ability of recombinant, flag-tagged $CtBP_L$ expressed in a ubiquitous fashion, to dimerize with endogenous $CtBP_L$ and CtBPs. The recombinant wild type $CtBP_L$ effectively and specifically immunoprecipitated endogenous long and short proteins, indicating that the $CtBP_L$ can homo- and hetero-dimerize (Figure III-4A). Coimmunoprecipitations with recombinant $CtBP_S$ also co-precipitated both endogenous isoforms comparable to recombinant $CtBP_L$, indicating that both tailed and untailed forms of CtBP are capable of forming dimers, indicating that dimer formation is characteristic of all CtBP proteins (Figure III-4B). Thus, the low expression levels of CtBP proteins do not appear to be reflective of a lack of dimerization potential.

Figure III-4: Immunoprecipitation of endogenous $CtBP_L$ and $CtBP_S$ with FLAG-tagged CtBP proteins indicate CtBP homo- and heterodimerization

A. Embryo extracts prepared from transgenic lines that express recombinant $CtBP_L$ (FLAG-tagged) from the CtBP native promoter were immunoprecipitated with anti-M2 (lane 5) and anti-IgG as a negative control (lane 7). Endogenous CtBP isoforms specifically co-precipitated with the recombinant protein, and no non-specific associations were observed with either beads alone (lane 6) or IgG (lane 7). A sample of non-transgenic embryo extract (yw EE) was loaded as a size marker to judge migration of CtBP proteins (lane 4).

B. Embryo extracts prepared from transgenic lines that express recombinant $CtBP_S$ (FLAG-tagged) from CtBP native promoter were immunoprecipitated with anti-M2 (lane 5). Endogenous CtBP isoforms specifically co-precipitated with the recombinant protein, and no non-specific associations were observed with either beads alone (lane 6) or anti-M2 IP of a non-transgenic embryo extract (lane 4). A sample of non-transgenic embryo extract (yw EE) was loaded as a size marker to judge migration of CtBP proteins (lane 7).

A.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Ptn-G Beads
anti-IgG IP
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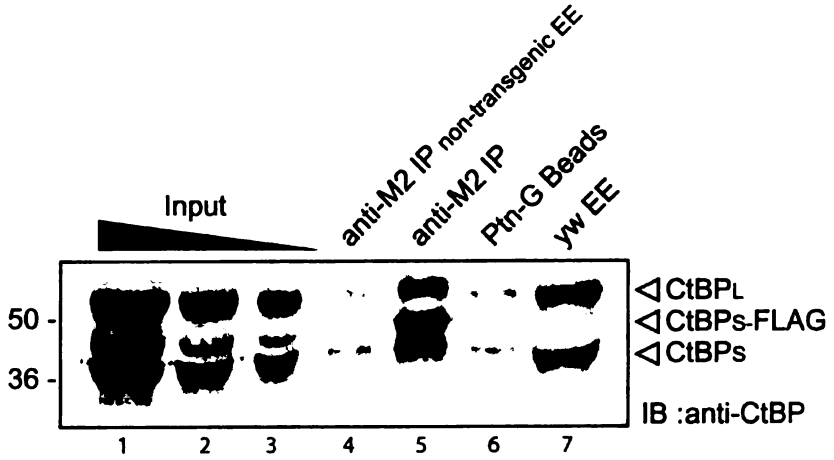
36 -
CtBPL-FLAG
CtBPL
CtBPs
IB :anti-CtBP
1 2 3 4 5 6 7

B.


Input
anti-M2 IP non-transgenic EE
anti-M2 IP
Ptn-G Beads
yw EE
50 -
36 -
CtBPL
CtBPs-FLAG
CtBPs
IB :anti-CtBP
1 2 3 4 5 6 7

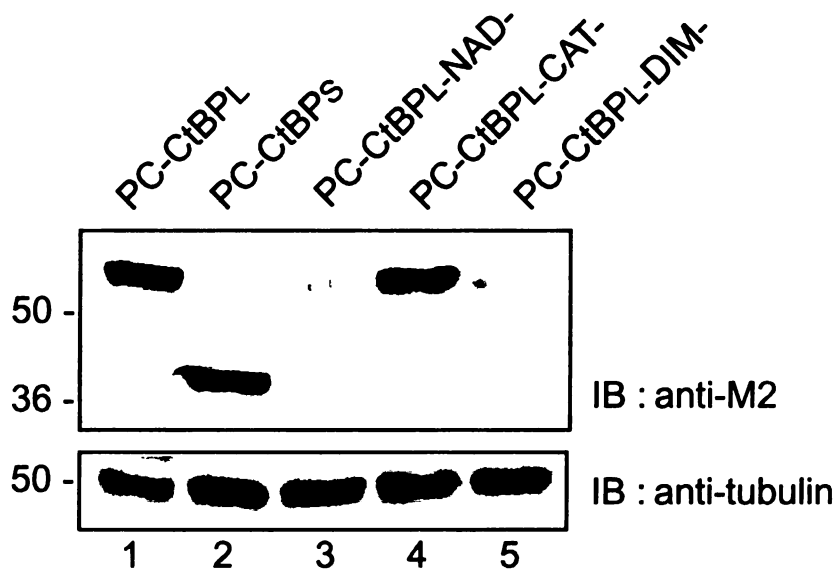


Figure III-5: $CtBP_L$ (NAD) and $CtBP_L$ (DIM) mutants are expressed at low steady state levels

Total extracts from single flies were prepared from transgenic lines that express recombinant wild type or putative dehydrogenase mutants from CtBP promoter (PC-CtBP). Extracts were analyzed by immunoblotting with anti-M2. Consistent with low levels of $CtBP_L$ (NAD) or $CtBP_L$ (DIM) observed in misexpression experiments, Figure III-2, we observed low expression of these mutants when driven from the native CtBP promoter as well (lanes 3 and 5). In comparison to low levels of protein observed in misexpression experiments, $CtBP_S$ was clearly well expressed in this context (lane 2). The bottom panel shows the same blot reprobed with β-tubulin as a loading control.

$CtBP_L$ (NAD) binding mutant is not defective for dimer formation

NAD binds to a central region of CtBP that has been postulated to mediate an allosteric modulation of the protein allowing dimer formation and in vitro, proteolytic accessibility of the protein changes in the presence of NAD (Kumar et al., 2002; Zhang et al., 2002). Data from the crystal structure of vertebrate CtBP1 suggests that residues important for dimerization largely overlap with the central NAD binding region (Kumar et al., 2002). In light of our results with low expression levels of $CtBP_L$ (NAD) or $CtBP_L$ (DIM), we thought it likely that both these mutants are affected similarly, namely loss of NAD binding might prevent dimerization through an allosteric mechanism, resulting in the loss of dimerization, just as mutating key residues in the dimer interface might block physical association. In both these circumstances, we hypothesized that the inability to form dimers might ultimately lead to instability of proteins that might account for low steady state protein levels observed.

To test this hypothesis, we sought to study the mutant *Drosophila* proteins. In contrast to the presumptive catalytic mutant, the NAD binding and dimerization mutants were expressed at low steady-state levels both when overexpressed using the Gal4-driver as well as when expressed using the 8Kb CtBP regulatory region. This low expression level limits our ability to physically characterize the protein produced in transgenic flies (Figure III-5). Therefore, to determine if these mutants are compromised for dimer formation, we expressed the wild type and mutants as His- and Flag-tagged proteins in bacteria (~64 KDa), purified them over a Ni^{2+}-NTA matrix and

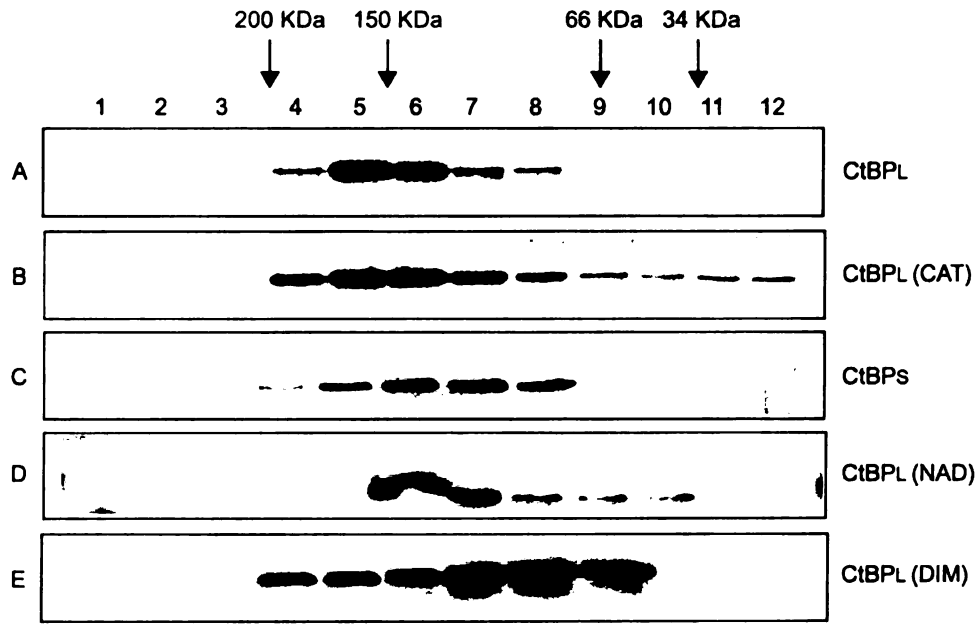


Figure III-6: NAD binding mutant migrates in molecular mass consistent with dimers but not the dimerization mutant

Peak fractions from Superdex 200 size exclusion experiments with wild type CtBP and mutants were resolved on SDS-PAGE gels and western blotted with anti-M2 antibody (to detect FLAG-tagged purified proteins). A pool of 3 fractions was loaded per lane. The $CtBP_L$ (CAT), $CtBP_S$ and $CtBP_L$ were found to migrate as dimers (~150 KDa) consistent with their ability to associate with partner CtBPs (Also see coimmunoprecipitations with $CtBP_S$ and $CtBP_L$ in Figure III-3). The $CtBP_L$ (DIM) (his-tagged and FLAG-tagged) was seen to peak in fractions in accordance with monomers. Significantly, the $CtBP_L$ (NAD) mutant was observed in fractions corresponding to dimers, suggesting that this mutant is able to associate with other CtBP proteins. Size markers were resolved under the same conditions and are indicated in the figure.

analyzed them by gel filtration chromatography. When fractions were analyzed by immunoblotting, $CtBP_L$ was observed to migrate in fractions compatible with the size of a dimer, having a molecular mass of about 150 KDa, consistent with size exclusion results of recombinant vertebrate CtBP1 by others (Balasubramanian et al., 2003; Nardini et al., 2003). The presumptive catalytic mutant that has activity comparable to the wild type $CtBP_L$, had a similar elution profile as $CtBP_L$. $CtBP_S$ (~50 KDa) eluted in slightly later fractions, but with a migration pattern consistent with dimer formation, in accordance with our coimmunoprecipitation results. Unlike the others, the $CtBP_L$ (DIM) mutant predominantly migrated in fractions consistent with monomers. Interestingly, the $CtBP_L$ (NAD) mutant was observed to elute in fractions of higher molecular mass similar to the profile of wild type CtBP, suggesting that mutation of residues critical for NAD binding does not disrupt dimerization.

DISCUSSION

The role of CtBP as an essential corepressor protein has been well established but the significance of its dehydrogenase-like domain is still unclear (Chinnadurai, 2006a). Previous studies aiming at similar questions have relied upon cell culture techniques where contrasting results have been obtained. Here, we sought to tease apart the contributions of its NAD binding abilities, dimerization and its putative active site in the context of the whole organism.

Dimerization is essential for CtBP functions

In our hands, misexpression of wild type full-length CtBP results in developmental abnormalities, but interestingly only in specific settings. The constitutive expression of CtBP proteins from both the tubulin promoter and the CtBP promoter did not disrupt development, suggesting that such outcomes are dependent on certain thresholds of misexpression that were achieved using strong tissue specific drivers. The phenotypic consequences of excessive corepressor activity may be due to direct repression of endogenous targets by CtBP in eye/wing tissues, or indirect effects resulting from the titration of cofactors required for regulation of other genes in these tissues.

Repression mediated via CtBP might be established by associated cofactors, with CtBP functioning as a tether to connect these repressive activities to target promoters. Alternatively, CtBP may employ its putative catalytic activity to mediate repression. A weak dehydrogenase activity has

been associated with vertebrate CtBP1, but the significance of this enzymatic activity warrants further investigation into physiological substrates and function (Balasubramanian et al., 2003; Kumar et al., 2002). Mutations affecting the presumptive catalytic histidine analogous to those used to inactivate dehydrogenases, did not impair the biological activity of CtBP as shown here or its repression ability seen earlier (Sutrias-Grau and Arnosti, 2004). Analysis of the presumptive catalytic mutant shows that it can associate effectively with other CtBP proteins; this attribute of dimerization might not only likely mask the outcome of the catalysis defect but also explains our misexpression phenotypes obtained with this mutant. However, this putative catalytic residue is strictly conserved in metazoan CtBP proteins and strongly speaks for conservation in function. Thus, the enzymatic function if any, might pertain to either transcriptional repression or other cellular functions. I propose that a role for this conserved residue might be best investigated in a CtBP null context.

Dimerization amongst CtBP proteins appears to be a conserved concept. Wild type isoforms of *Drosophila* CtBP can homo- and heterodimerize in vivo. This dimer formation might form the mechanistic basis for CtBP to nucleate of a repressive complex, with one monomer attaching to the DNA bound repressor and the other monomer allowing its PXDLS site to be contacted by repressive cofactors. CtBP may thus use its dimerization ability to tether transcriptional repression activities to target promoters.

NAD binding and function

Substitutions of key glycines in the central NAD binding domain in CtBP have been shown to inhibit NAD binding (Kumar et al., 2002; Nardini et al., 2003). Our misexpression analyses with a $CtBP_L$ (NAD) mutant revealed low steady state levels of this protein. Previous reports analyzing this mutant fused to a Gal4 tether found a complete loss of transcriptional repression and stable protein levels *in vivo* (Sutrias-Grau and Arnosti, 2004). The presence of a heterologous DNA binding domain might help stabilize this protein by directly tethering it to the promoter. The $CtBP_L$ (DIM) mutant also exhibited low steady state levels. In light of prior studies that suggest that NAD binding by CtBP allows oligomerization (Balasubramanian et al., 2003), we speculated that the reason for low steady state levels of the $CtBP_L$ (NAD) and $CtBP_L$ (DIM) mutants might be a consequence of inability to dimerize. Our size exclusion results with recombinant versions of these mutants raised in bacteria showed that the NAD mutant was capable of migrating in fractions consistent with dimers, even in the absence of NAD. This profile is in sharp contrast to the DIM mutant that elutes predominantly in fractions consistent with monomers. The $CtBP_L$ (NAD) elution profile is also somewhat different from that observed for $CtBP_L$, suggesting that the protein might adapt a different conformation perhaps due to lack of NAD binding. Clearly, this dimerization is not enough to rescue the transcriptional repression function of this mutant when this mutant was tested for repression of an integrated reporter. These results also rule out the earlier speculation that an inability to dimerize might be responsible for low steady state levels of protein observed

in vivo. We speculate that in spite of dimer formation, the absence of NAD binding prevents these dimers from undergoing a conformational change that excludes binding to cofactors. This might be one plausible explanation for the low steady state levels and loss of repression observed.

Future Considerations

Relevant to this idea that NAD induced conformational change is needed to contact transcriptional cofactors, I propose to perform coimmunoprecipitations to test for the association of candidate cofactors like LSD1, Rpd3 and a DNA binding repressor like Krüppel, with wild type vs. the NAD binding and DIM mutants. A complementary approach will be to compare the migration properties of epitope tagged CtBP wild type and mutants generated in *Drosophila* by gel filtration chromatography. Here, the ability of CtBP to migrate with cofactors will be tested. If the low steady state levels of the $CtBP_L$ (NAD) mutant are indeed a result of lack of association with cofactors, this mutant will be seen to migrate in fractions of lower molecular weight and the $CtBP_L$ (DIM) mutant will adequately serve as a control.

It has been documented that in the presence of NAD+/NADH, wild type $CtBP_L$ migration on a sizing column is seen to shift to higher order oligomers . Experiments with recombinant $CtBP_L$ demonstrate that both forms of the nucleotide were effective in stimulating oligomerization of CtBP, which was seen to migrate in fractions of higher molecular weight (Balasubramanian et

al., 2003). This observation prompted us to test the migration characteristics of the recombinant $CtBP_L$ (NAD) mutant relative to the wild type $CtBP_L$, in the presence and absence of nucleotides. However, in our hands the elution profile of recombinant wild type $CtBP_L$ protein in the absence vs. presence of NAD+ remained unchanged (P.Mani, data not shown). Further experiments are warranted to test if the $CtBP_L$ (NAD) mutant is unresponsive to the effects of NAD and migrates in similar fractions even in the absence of NAD. If NAD induces a conformational change in the protein that enhances oligomerization, then the $CtBP_L$ (NAD) mutant should be completely unaffected, and its observed migration as dimers eluting at ~150 KDa should remain unchanged.

REFERENCES

Arnosti, D. N. (2004). Multiple Mechanisms of Transcriptional Repression in Eukaryotes. In *Handbook of Experimental Pharmacology*, vol. 166 (ed. Gossen M. Kaufmann J. and Triezenberg S.J.): Springer(Berlin).

Balasubramanian, P., Zhao, L. J. and Chinnadurai, G. (2003). Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. *FEBS Lett* **537**, 157-60.

Boyd, J. M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S. and Chinnadurai, G. (1993). A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *Embo J* **12**, 469-78.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol Cell* **9**, 213-24.

Chinnadurai, G. (2006a). CtBP family proteins:unique transcriptional regulators in the nucleus with diverse cytosolic functions: Landes Bioscience.

Chinnadurai, G. (2006b). CtIP, a candidate tumor susceptibility gene is a team player with luminaries. *Biochim Biophys Acta* **1765**, 67-73.

Corda, D., Colanzi, A. and Luini, A. (2006). The multiple activities of CtBP/BARS proteins: the Golgi view. *Trends Cell Biol* **16**, 167-73.

Criqui-Filipe, P., Ducret, C., Maira, S. M. and Wasylyk, B. (1999). Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation. *Embo J* **18**, 3392-403.

Furusawa, T., Moribe, H., Kondoh, H. and Higashi, Y. (1999). Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor deltaEF1. *Mol Cell Biol* **19**, 8581-90.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr Opin Cell Biol* **8**, 358-64.

Grooteclaes, M., Deveraux, Q., Hildebrand, J., Zhang, Q., Goodman, R. H. and Frisch, S. M. (2003). C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs. *Proc Natl Acad Sci U S A* **100**, 4568-73.

Hildebrand, J. D. and Soriano, P. (2002). Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol* **22**, 5296-307.

Imai, S., Armstrong, C. M., Kaeberlein, M. and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795-800.

Kegel, K. B., Meloni, A. R., Yi, Y., Kim, Y. J., Doyle, E., Cuiffo, B. G., Sapp, E., Wang, Y., Qin, Z. H., Chen, J. D. et al. (2002). Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. *J Biol Chem* **277**, 7466-76.

Keller, S. A., Mao, Y., Struffi, P., Margulies, C., Yurk, C. E., Anderson, A. R., Amey, R. L., Moore, S., Ebels, J. M., Foley, K. et al. (2000). dCtBP-dependent and -independent repression activities of the Drosophila Knirps protein. *Mol Cell Biol* **20**, 7247-58.

Kim, J. H., Cho, E. J., Kim, S. T. and Youn, H. D. (2005). CtBP represses p300-mediated transcriptional activation by direct association with its bromodomain. *Nat Struct Mol Biol* **12**, 423-8.

Koipally, J. and Georgopoulos, K. (2000). Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity. *J Biol Chem* **275**, 19594-602.

Kumar, V., Carlson, J. E., Ohgi, K. A., Edwards, T. A., Rose, D. W., Escalante, C. R., Rosenfeld, M. G. and Aggarwal, A. K. (2002). Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell* **10**, 857-69.

Nardini, M., Spano, S., Cericola, C., Pesce, A., Massaro, A., Millo, E., Luini, A., Corda, D. and Bolognesi, M. (2003). CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. *Embo J* **22**, 3122-30.

Rutter, J., Reick, M., Wu, L. C. and McKnight, S. L. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**, 510-4.

Schmitz, F., Konigstorfer, A. and Sudhof, T. C. (2000). RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron* **28**, 857-72.

Senyuk, V., Sinha, K. K. and Nucifora, G. (2005). Corepressor CtBP1 interacts with and specifically inhibits CBP activity. *Arch Biochem Biophys* **441**, 168-73.

Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P., Nakatani, Y. and Shi, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**, 735-8.

Struffi, P. and Arnosti, D. N. (2005). Functional interaction between the Drosophila knirps short range transcriptional repressor and RPD3 histone deacetylase. *J Biol Chem* **280**, 40757-65.

Struffi, P., Corado, M., Kulkarni, M. and Arnosti, D. N. (2004). Quantitative contributions of CtBP-dependent and -independent repression activities of Knirps. *Development* **131**, 2419-29.

Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol* **24**, 5953-66.

Thio, S. S., Bonventre, J. V. and Hsu, S. I. (2004). The CtBP2 co-repressor is regulated by NADH-dependent dimerization and possesses a novel N-terminal repression domain. *Nucleic Acids Res* **32**, 1836-47.

Zhang, H. and Levine, M. (1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo. *Proc Natl Acad Sci U S A* **96**, 535-40.

Zhang, Q., Piston, D. W. and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895-7.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Drosophila serves as an exemplary model to study the spatial control of gene activity in development, during which boundaries of gene expression are resolved by spatially restricted activity or localization of transcriptional repressors (Mannervik et al., 1999). Studies on transcriptional repression in the *Drosophila* embryo have identified two basic modes of repression: long-range and short-range repression (Gray and Levine, 1996). Short-range repression represents a more flexible, locally acting kind of gene regulation where repressors are documented to primarily act by 'quenching' of activator proteins or directly repress basal machinery components (Arnosti, 2004; Hewitt et al., 1999). In contrast, long-range repressors function more globally blocking multiple enhancers in a vicinity of several kilobases. As discussed in Chapter I, short-range repressors work with the evolutionarily conserved corepressor protein CtBP through a consensus sequence while long-range repressors mediate their effect through the corepressor Groucho. The different activities of of each repressor class are believed to reflect distinct mechanisms at play; however the molecular events after corepressor recruitment are not clearly understood. Some long-range repressors are also seen to interact with CtBP and the role of CtBP in this context is ambiguous. For instance, CtBP binding to Hairy has been shown to antagonize repression while in the context of Runt long-range repressor, CtBP is apparently needed for maintaining repression of Runt targets in the early embryo (Phippen et al., 2000; Wheeler et al., 2002).

CtBP is a major contributor to the activity of short-range repressors during development and has been demonstrated to modulate the activities of oncogenes, signaling pathways and apoptosis (Chinnadurai, 2006). The precise mechanism of action of CtBP has been under intense scrutiny both in vertebrate and in invertebrate model organisms.

$CtBP_L$ and $CtBP_S$ - developmentally regulated roles

In vertebrates, multiple genes for *CtBP* are seen to encode for splice variants that execute divergent functions (Discussed in Chapter I). The fly *CtBP* gene also encodes for two major isoforms ($CtBP_L$ and $CtBP_S$) of unidentified functions. Both forms of the proteins show comparable repression potential (Sutrias-Grau and Arnosti, 2004). $CtBP_L$ differs from $CtBP_S$ by possessing an unstructured C-terminal tail that is a target for several post-translational modification events. My analysis of isoform expression reveals a conserved developmental shift in the levels of $CtBP_L$ isoform at later stages (Mani-Telang and Arnosti, 2006). This is a first demonstration highlighting temporal expression differences between these splice variants and provokes the key question regarding isoform specific needs for specific functions during development. The observation that genomes of related Dipterans and other organisms from Hymenoptera and Coleoptera, all contain $CtBP_L$ coding information and show the presence of multiple isoforms, suggests that the existence of multiple isoforms is a widely conserved trait, and may have important functional ramifications. My data is in accordance with *in vivo* mice

CtBP knockouts (Hildebrand and Soriano, 2002). Mice null for *CtBP1* and *CtBP2* suggest unique and overlapping phenotypes, different isoforms are regulated through different post-translational modifications; lending support to the hypothesis of different isoforms having specific functions.

Mechanisms behind the observed down-regulation of $CtBP_L$

As described earlier, developmental study of isoform expression reveals a conserved shift in the levels of $CtBP_L$ isoform at later stages. The evident drop in $CtBP_L$ protein levels can be traced back to transcript abundances. My investigations of relative transcript levels by RT-PCR measurements indicate that $CtBP_S$ mRNA is upregulated relative to $CtBP_L$ at later developmental times, which is mirrored in the protein profile with $CtBP_S$ protein being more predominant in adults.

In addition to splicing preferences, the decrease in $CtBP_L$ levels may also be a consequence of regulatory post-translational modification events that target the unique C-terminus of the protein. Numerous such modifications have been identified that act on individual vertebrate isoforms and serve to regulate isoform localization (availability) or abundance (discussed in Chapter 1-Section 1.3). SUMOylation of vertebrate CtBP1 is required for its nuclear translocation and disrupting this modification has a profound effect on CtBP's localization and corepressor function (Lin et al., 2003). My bioinformatic analysis comparing sequence information for $CtBP_L$ tail among highly divergent organisms illustrates that SUMOylation motifs are preserved (See

Chapter II - Figure II-2). Previous demonstration of SUMOylation affecting vertebrate CtBP function and the conservation of SUMO motifs in divergent organisms suggests SUMO regulation of CtBP must be operative in *Drosophila* as well. It will be interesting to determine the role of SUMOylation on CtBP function in a developmental setting. Since SUMOylation is known to alter the localization of CtBP1, the subcellular localization of tagged protein forms can also be tested by transfections in *Drosophila* cells.

CtBP proteins are also phosphorylated; one such phosphorylation event by HIPK2 kinase at Ser-422 in the C-terminus results in ubiquitination of vertebrate CtBP followed by proteasomal clearance, which can be prevented by proteasomal inhibitor treatment (Zhang et al., 2005; Zhang et al., 2003). Preliminary evidence with the proteasomal inhibitor MG-132 shows that in *Drosophila* S2 cells, $CtBP_L$ levels can be stabilized in comparison to $CtBP_S$ levels, that stay constant (Y. Zhang, unpublished results). The C-termini of vertebrate vs. fly CtBP proteins differ in sequence, but are conserved in overall length and are abundant in disorder promoting residues (P.Mani, data not shown). In this region of *Drosophila* CtBP, several serine residues exist that could serve as potential phosphorylation sites. Analysis of truncated derivatives of the tail region that eliminate SUMOylation and phosphorylation sites will help shed light on the importance of these modifications. $CtBP_S$ lacks this stretch of the C-terminus and is the predominantly expressed protein at all developmental times; in light of our developmental expression data, it will prove to be interesting to test if truncated $CtBP_L$ derivatives are converted to more stable proteins. Taking these studies one step further, the localization of these truncated derivatives can be studied. It is likely that a deletion disrupting

the conserved SUMO motifs from *Drosophila* $CtBP_L$ might result in its translocation to the cytoplasm. Along the same lines, it will be interesting to study the repression activity of these deletion mutants as translocation of CtBP into the cytoplasm will interfere with its corepressor functions.

Functional equivalency of CtBP isoforms in *Drosophila*

Understanding the biological functions of CtBP isoforms has been a major challenge. In previous studies, comparing $CtBP_L$ and $CtBP_S$ activity, no differences have been uncovered (Fang et al., 2006; Sutrias-Grau and Arnosti, 2004). The observed decline in *Drosophila* $CtBP_L$ levels are thus very interesting and could be interpreted as $CtBP_S$ furnishing $CtBP_L$ like functions in later stages. It is possible that $CtBP_L$ function is no longer required during these developmental periods and is hence down regulated, in which case genes targeted specifically by $CtBP_L$ should be derepressed. One motive for the misexpression strategy was to compare the biological activity of $CtBP_L$ and $CtBP_S$. However, due to low expression levels of the short isoform the results were inconclusive. Further analysis using coimmunoprecipitations and size exclusion chromatography demonstrated that $CtBP_S$ proteins and $CtBP_L$ proteins can homo- and heterodimerize. This observation tells us that an appropriate assessment of individual isoform function can only be provided in a CtBP null background, where dimerization is not feasible.

To determine if these isoforms are functionally redundant or exist for unique function, a whole animal assay of CtBP activity is proposed. As

mentioned earlier, the motivation behind the experiment is to be able to assay individual isoform activity in a CtBP null backdrop. In *Drosophila*, homozygous mutations in *CtBP* are lethal. I identified the endogenous regulatory region required for expression of CtBP and created transgenic lines expressing $CtBP_L$ and $CtBP_S$ from this endogenous CtBP promoter. My initial attempts to rescue this lethality using transgenes for $CtBP_L$ alone, $CtBP_S$ alone or both did not suffice. Several reasons for this lack of replacement are discussed in detail in the following section (Appendix A). Instead of a transgene based rescue, a more sureshot way of assaying for function is to attempt a genomic rescue. In this experiment, the entire genomic locus including all the regulatory information for *CtBP* will be introduced into flies, and assessed for its ability to rescue CtBP nulls. A successful rescue will permit us to make modifications in this genomic locus (for e.g. block splice site that generates $CtBP_L$) and answer the original question regarding whether the fly can survive on the basis of any one isoform.

Structure- function studies

CtBP proteins resemble dehydrogenases in having a conserved Rossman fold required for NAD binding and a catalytic triad (His/Glu/Arg) conserved in all enzymes where the histidine forms the active site center (Chinnadurai, 2002). Consistent with the presence of the Rossman fold, vertebrate CtBP1 and 2 have been documented to bind with NAD+ and NADH, with contrasting views on which metabolite has a higher affinity for CtBP (Kumar et al., 2002; Zhang et al., 2002). Based on these views, it has been suggested that CtBP may regulate transcription in a redox-responsive manner (Zhang et al., 2002). In addition, a weak enzymatic activity has been noted with CtBP1, which adds a layer of complexity to solving the mechanism of CtBP-mediated repression. Previous work has suggested that NAD binding promotes multimerization of the protein in vitro (Balasubramanian et al., 2003) and possibly allows interaction with PXDLS-containing cofactors by inducing a conformational alteration in the protein (Kumar et al., 2002). However, the temporal sequence of molecular events has not been tested. It is not known if dimerization is required for cofactor association, if the NAD induced conformational change is mandatory for cofactor binding or if individual CtBP monomers are already bound to cofactors and are recruited through dimerization with another CtBP monomer, associated promoter bound repressor.

Since these structural features are intact in the fly CtBP proteins, we initiated studies on the relevance of these features by making mutations in conserved residues and assaying them in a whole animal context. Our NAD

binding mutant was ineffective as a corepressor while a catalytic site deficient mutant retained full repressive potential, giving us the first indication that NAD binding is essential for repression (Sutrias-Grau and Arnosti, 2004). My misexpression studies with the wild type and mutant proteins also painted a consistent picture with the catalytic mutant showing comparable activity as wild type $CtBP_L$; however, the NAD binding mutant was expressed poorly.

When tested for dimerization, $CtBP_L$, $CtBP_S$ and the catalytic mutant were able to dimerize. Since these proteins were also seen to retain repressive abilities, it is likely that dimerization allows for cofactors to be recruited. A stepwise recruitment model can be envisioned wherein a DNA binding repressor may recruit CtBP as a corepressor. CtBP may then dimerize (or bind as a dimer), and then use its dimer partner to recruit repressive cofactors. Towards this aim, I generated a dimerization mutant that also yielded the same results as the NAD binding mutant and was poorly expressed. Since the residues for dimerization map along the NAD binding region (Kumar et al., 2002), it was my hypothesis that probably the reason these proteins are expressed poorly is because of defects in CtBP dimerization. I speculated that the NAD binding mutant cannot bind NAD and hence cannot undergo the conformational change that allows for dimerization, while the dimerization mutant can bind NAD but is restricted by mutations in critical residues needed to contact its dimer partner.

Since the NAD binding and the dimerization mutant were both poorly expressed in flies (with several promoter constructs), I generated purified recombinant proteins in bacterial and fractionated them by size exclusion chromatography. The wild type $CtBP_L$ and $CtBP_S$ pure proteins resolved in

fractions of about 150KDa, indicative of dimers. I observed the catalytic mutant to migrate in similar size fractions and coimmunoprecipitate with the wild type proteins, suggesting that this mutant could associate with another CtBP monomer. This result explains my misexpression data and repression data from our previously published study, wherein the repressive activity of this mutant could perhaps be a result of association with endogenous CtBP proteins. This result emphasizes that in order to be able to ascribe a role for the dehydrogenase activity associated with CtBP, one needs to evaluate it in a CtBP null background. In accordance with my hypothesis, the dimerization mutant peaked in monomeric fractions. This is a first demonstration of the inability of this mutant to dimerize. However, this mutant has been shown to be unable to contact a PXDLS partner, E1A in cell culture based studies suggesting that the protein will be ineffective as a repressor (Kumar et al., 2002). It will be interesting to test if this mutant is active for repression in *Drosophila*. If the hypothesis that dimerization preceded cofactor recruitment is valid, then this mutant is predicted to fail as a repressor.

Surprisingly, our hypothesis with the NAD binding mutant proved to be incorrect. This mutant was seen to migrate in fractions consistent with dimers of CtBP, suggesting that NAD binding is not mandatory for associating with a dimer partner, as has been speculated. This leaves open the question of why the NAD binding mutant is expressed poorly and why does it lack repressive function. My speculation is that perhaps dimers formed in the absence of NAD are not functional. NAD acts as a switch to bring about a change in conformation of CtBP; it is possible that monomers of CtBP can stick to each other in the absence of NAD, but their PXLDS responsive clefts are not

exposed/available. This might prohibit interaction with cofactors and is a likely explanation for the lack of repression observed.

I will test this hypothesis by performing coimmunoprecipitations, similar to those described in Chapter III, to probe for a lack of cofactor associations with the NAD binding and the dimerization mutant. Recombinant $CtBP_L$ proteins expressed in flies are seen to associate with a histone demethylase LSD1 and BAF53 in coimmunoprecipitation studies. Using extracts from flies expressing the NAD binding and dimerization mutants, I will investigate whether interaction with these cofactors is abolished. Another important step leading to repression is the recruitment of CtBP corepressor by DNA binding proteins to target promoters. I shall also determine if the mutants can associate with a DNA binding repressor like Knirps or Kruppel (short-range repressors in the fly). As a complementary approach, fly extracts expressing wild type CtBP versus the NAD binding and dimerization mutants will be fractionated on a sizing column; here I will be assaying the ability of these proteins to traverse in high molecular weight fractions indicative of a CtBP complex, containing its suite of repressive cofactors. In light of previous studies, the dimerization mutant should serve as a control for non-specific DNA mediated interactions, by migrating as monomeric units, if the hypothesis is accurate. These complementary approaches focusing on the relevance of NAD binding and dimerization will throw light on CtBP-mediated molecular mechanisms of repression.

In conclusion, the *Drosophila* CtBP protein is regulated in more ways that currently known. The studies described herein provide insights into two very crucial aspects of CtBP mediated repression during development –

1) Its conserved developmental regulation strongly suggests distinct functions during development.

2) NAD binding is not a pre-requisite for dimerization of CtBP proteins, but may be required for cofactor association.

REFERENCES

Arnosti, D. N. (2004). Multiple Mechanisms of Transcriptional Repression in Eukaryotes. In *Handbook of Experimental Pharmacology*, vol. 166 (ed. K. J. a. T. S. J. Gossen M.): Springer(Berlin).

Balasubramanian, P., Zhao, L. J. and Chinnadurai, G. (2003). Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. *FEBS Lett* **537**, 157-60.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol Cell* **9**, 213-24.

Chinnadurai, G. (2006). CtBP family proteins:unique transcriptional regulators in the nucleus with diverse cytosolic functions: Landes Bioscience.

Fang, M., Li, J., Blauwkamp, T., Bhambhani, C., Campbell, N. and Cadigan, K. M. (2006). C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. *Embo J* **25**, 2735-45.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr Opin Cell Biol* **8**, 358-64.

Hewitt, G. F., Strunk, B. S., Margulies, C., Priputin, T., Wang, X. D., Amey, R., Pabst, B. A., Kosman, D., Reinitz, J. and Arnosti, D. N. (1999). Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient. *Development* **126**, 1201-10.

Hildebrand, J. D. and Soriano, P. (2002). Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol* **22**, 5296-307.

Kumar, V., Carlson, J. E., Ohgi, K. A., Edwards, T. A., Rose, D. W., Escalante, C. R., Rosenfeld, M. G. and Aggarwal, A. K. (2002). Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell* **10**, 857-69.

Lin, X., Sun, B., Liang, M., Liang, Y. Y., Gast, A., Hildebrand, J., Brunicardi, F. C., Melchior, F. and Feng, X. H. (2003). Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Mol Cell* **11**, 1389-96.

Mani-Telang, P. and Arnosti, D. N. (2006). Developmental expression and phylogenetic conservation of alternatively spliced forms of the C-terminal Binding Protein corepressor. *Development, Genes and Evolution* **In Press**.

Mannervik, M., Nibu, Y., Zhang, H. and Levine, M. (1999). Transcriptional coregulators in development. *Science* **284**, 606-9.

Phippen, T. M., Sweigart, A. L., Moniwa, M., Krumm, A., Davie, J. R. and Parkhurst, S. M. (2000). Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression. *J Biol Chem* **275**, 37628-37.

Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol* **24**, 5953-66.

Wheeler, J. C., VanderZwan, C., Xu, X., Swantek, D., Tracey, W. D. and Gergen, J. P. (2002). Distinct in vivo requirements for establishment versus maintenance of transcriptional repression. *Nat Genet* **32**, 206-10.

Zhang, Q., Nottke, A. and Goodman, R. H. (2005). Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis. *Proc Natl Acad Sci U S A* **102**, 2802-7.

Zhang, Q., Piston, D. W. and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895-7.

Zhang, Q., Yoshimatsu, Y., Hildebrand, J., Frisch, S. M. and Goodman, R. H. (2003). Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP. *Cell* **115**, 177-86.

APPENDIX A

RESCUE OF CtBP FUNCTION

CtBP function is crucial for the regulation of early gene expression in *Drosophila*. Loss-of-function mutations in the gene are homozygous lethal with lethality occurring in during the late larval to early pupal stages (Poortinga et al., 1998) . Studies on vertebrate CtBP proteins have provided much data on the potential roles of CtBP in cell culture, but these studies have not shed light on the role of CtBP during development. *Drosophila* serves as an excellent model to perform whole organism genetic manipulations with ease.

The CtBP gene encodes for two major splice forms, which are both detected throughout all developing stages of the fly (Chapter I, Figure I-3) (Sutrias-Grau and Arnosti, 2004). However, there is a marked reduction in levels of $CtBP_L$ isoform from larval through adult stages, unlike $CtBP_S$ levels that stay relatively constant. I found that the changing abundance of distinct mRNA spliceforms might be the determinants of the developmental changes in protein profile. In addition, post-translational modifications might regulate overall levels of this protein (Mani-Telang and Arnosti, 2006). It is unknown if different isoforms provide distinct functions during development. The splice variants differ by a C-terminal extension present in $CtBP_L$ and in its place, $CtBP_S$ has a short stretch of 10 aa. Alternative splicing might provide specificity of CtBP functions during fly development. Because $CtBP_L$ is virtually identical to all CtBP proteins except for the C-terminal residues (similar in length, but vary in sequence) it is possible that this form of the protein can provide all functions of CtBP. On the other hand, a developmental

profile shows $CtBP_S$ to be the prevalent protein during the life cycle of the fly (Chaper II-Figure II-1C). In order to test potential redundancies between isoforms, I tested the ability of $CtBP_L$ and $CtBP_S$ to rescue a null mutant. This assay serves as a rigorous test of individual isoform function in light of our previously described analyses (Chapter II and III).

Two null mutations have been described for *CtBP*; the first is a P-element insertion in a 5'UTR of *CtBP* (#P-1590) (FigureA-1) and the second is a deletion around the same segment (#1663), both of which do not complement each other (Grumbling et al., 2006). I introduced a cDNA fragment for the long isoform into pCasper-tubulin vector, allowing expression under the control of a constitutively active tubulin promoter in flies (Basler and Struhl, 1994). This promoter is active early during *Drosophila* embryogenesis and protein expression can be detected ubiquitously by immunostaining, in a manner that mimics endogenous CtBP expression (data not shown). However, unlike the results obtained from tissue specific misexpression discussed in Chapter 3, the ubiquitous ectopic expression of the $CtBP_L$ isoform did not induce any phenotypes. Furthermore, I was unable to recover any survivors in a *CtBP-/-* mutant background. The failure to rescue might be attributed to the lack of compensation of the $CtBP_S$ isoform, insufficient levels or patterns of induced protein, or interference from the C-terminal flag-tag.

To faithfully mimic the endogenous CtBP expression levels and patterns *in vivo*, the genomic locus of CtBP was searched for regulatory information. The cis-regulatory elements governing CtBP transcription have not been previously explored. The entire locus is contained within a ~15 Kb

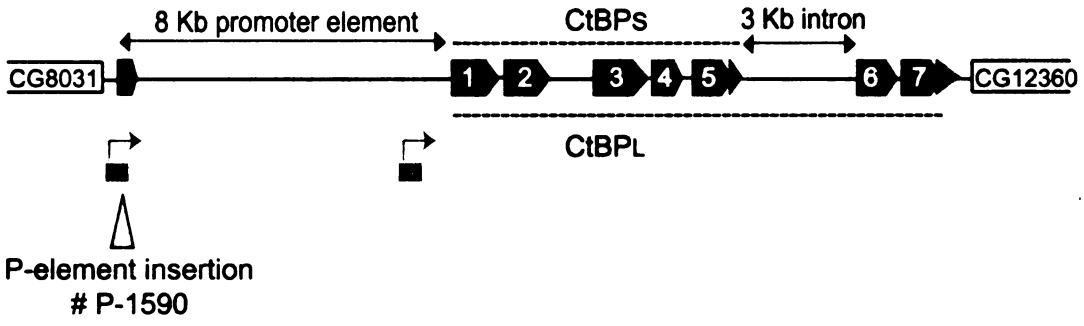


Figure A-1: Genomic organization of the *Drosophila CtBP* locus

CtBP gene locus extends over a ~15 Kb region and encodes two splice variants. Two predicted transcriptional start sites have been located, depicted as red boxes in the cartoon. A P-element insertion described for CtBP has been mapped to lie upstream of the coding sequence (Grumbling et al., 2006; Poortinga et al., 1998). An 8Kb genomic fragment 5' of the gene was used to drive expression of CtBP transgenes. Exons (black boxes) coding for $CtBP_L$ and $CtBP_S$ are indicated by dotted lines and occupy about 7 Kb of genomic sequence. The two additional exons that constitute the C-terminal tail of $CtBP_L$ are separated from the core CtBP sequences (exons 1-5) by a large intron of ~3 Kb that seems to be conserved in Drosophilids. $CtBP_S$ has a short 3' UTR of ~60 bp (light blue triangle) while $CtBP_L$ has a long ~700 bp UTR (green triangle).

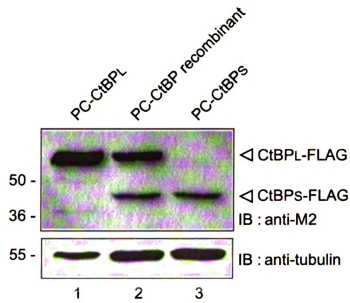


Figure A-2: Expression of CtBP isoforms under the control of endogenous promoter

An 8Kb upstream region directly upstream of the 5' most protein coding exon for CtBP was used to drive expression of FLAG-epitope tagged forms of $CtBP_L$ and $CtBP_S$. A line containing both of these transgenes was obtained by recombination (See Materials and Methods). Single adults from transgenic fly lines expressing these isoforms were solubilized in Laemmli sample buffer, loaded on 10% SDS-PAGE gels and analyzed by anti-M2 antibody to detect FLAG-tagged CtBP. Native promoter driven $CtBP_S$ is expressed at levels lower than $CtBP_L$, detected both by western blots and immunostaining of stage-specific embryos, which is in sharp contrast to the endogenous protein profile. The bottom panel shows β-tubulin as a loading control.

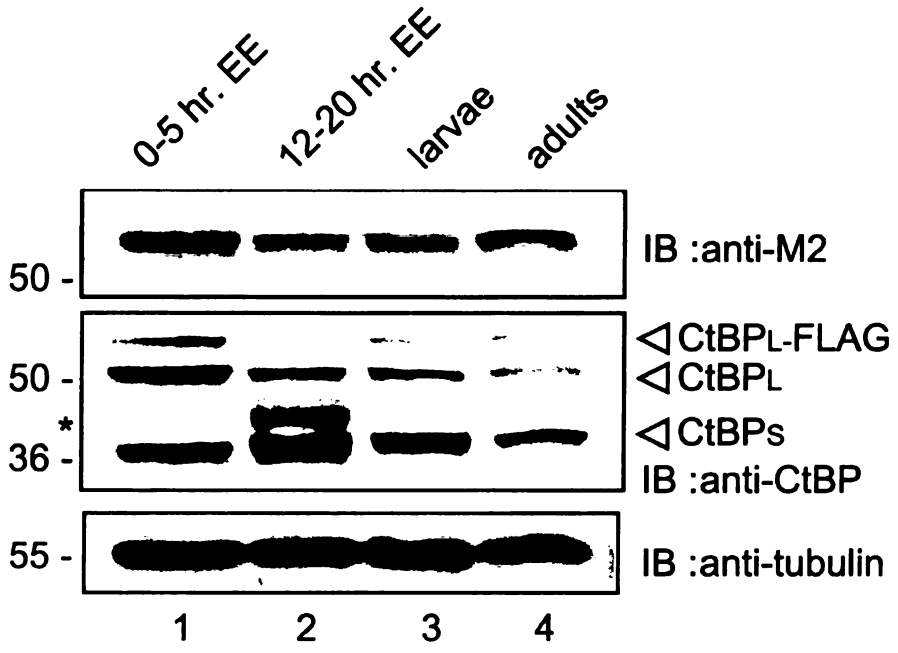


Figure A-3: Expression of recombinant $CtBP_L$ assayed by Western blotting

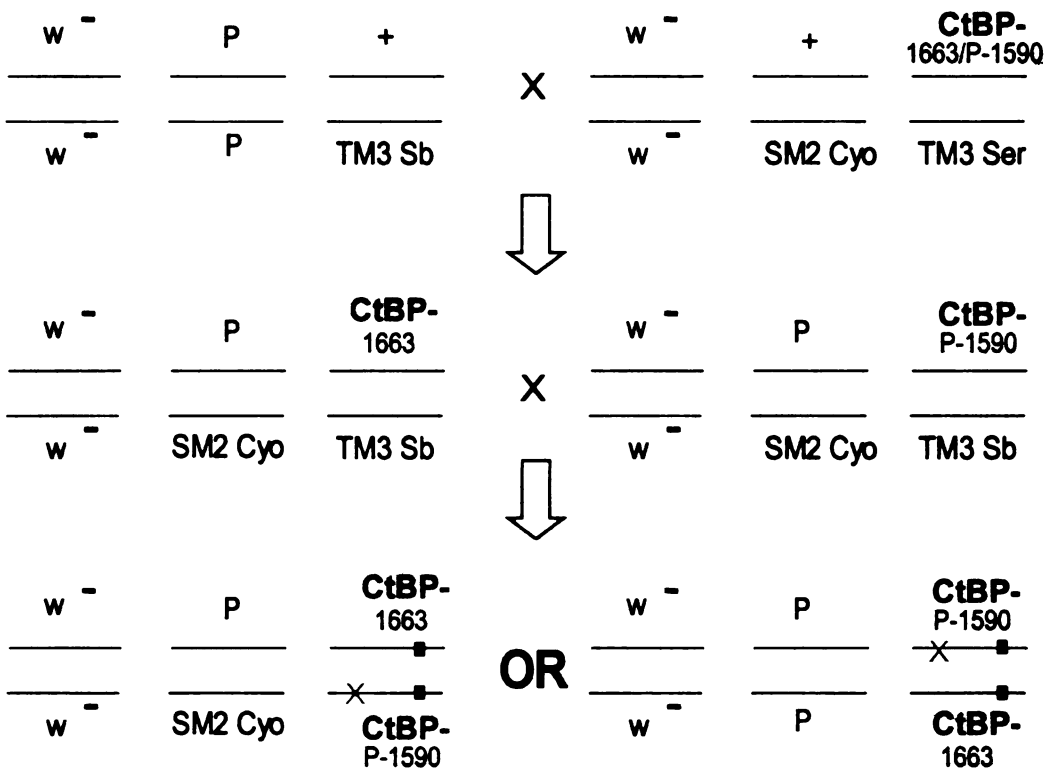
To determine if levels of recombinant FLAG-tagged $CtBP_L$ decrease at later developmental times as seen with endogenous $CtBP_L$, western blots were performed on stage-specific soluble extracts from transgenic fly lines expressing $CtBP_L$ under the CtBP promoter. Western blotting with anti-M2 (first panel) to detect FLAG-tagged $CtBP_L$, did not show any appreciable decrease in protein levels at later developmental stages, suggesting that the protein is not expressed at lower levels in larva and adult. However, when the same blot was stripped and reprobed with anti-CtBP antibody, a clear decrease in $CtBP_L$-FLAG similar to endogenous $CtBP_L$ was observed, suggesting that expression patterns (immunostaining, data not shown) and expression levels were comparable to native protein. The discrepancy between the two antisera could be on account of the CtBP antisera's limited cross-reactivity to modified forms of CtBP. The band marked with an asterisk in 12-20 hr. extract is unknown. The blot was stripped and reprobed again to show β-tubulin as a loading control.

region and the genomic organization seems to be conserved in Drosophilids (data not shown). Exons coding for CtBP are contained within ~6.8 Kb region with two predicted transcriptional start sites located -2 Kb and -8 Kb upstream of the translational initiation codon. In an effort to drive expression of CtBP using its endogenous regulatory elements, cDNA fragment specific to each isoform was expressed under the control of either -4 Kb or -8 Kb of genomic sequence (Figure A-1). The shorter 4 Kb 5' region did not induce detectable CtBP protein expression (data not shown). In contrast, the entire upstream region of ~8Kb yielded several transgenic lines which expressed CtBP, suggesting that ectopic expression from this promoter does not disrupt normal development (Figure A-2).

The rescue assay was devised to test for complementation in two steps: the first step tests rescue of the loss of zygotic CtBP (rescue of a homozygous null). If successful, the next stage tests if the rescued progeny can overcome the lack of both zygotic and maternally deposited CtBP. To test if $CtBP_L$ could compensate for endogenous CtBP functions, suitable transgenic lines (P-element insertion lines mapped to the second chromosome by standard genetic crosses) expressing CtBP promoter::$CtBP_L$-FLAG transgene under control of the native promoter was crossed into a CtBP mutant background (See Rescue scheme, Figure A-4). Such promoter driven transgene fusions have been effectively used for phenotypic rescue (Donaldson et al., 2004; Moran and Jimenez, 2006; Wagner et al., 2002).

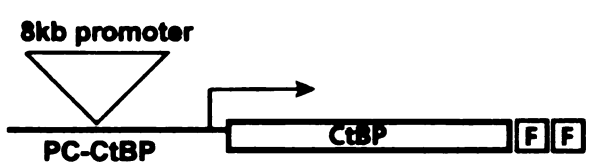
Figure A-4: Genetic crosses for whole animal rescue of CtBP mutant phenotype

#P-1590 is a P-element insertion that is mapped to an upstream 5' UTR in CtBP and #1663 is a deletion mutant in the same region and the two alleles do not complement each other (yellow boxes) (Grumbling et al., 2006). $CtBP_L$, $CtBP_S$ and a recombinant chromosome containing both the transgenes were assessed individually for rescue by crossing into different CtBP mutant backgrounds as shown in the first step of the scheme. Three chromosomes of *Drosophila* are drawn; with the CtBP mutations indicated the third chromosome (small black squares) and the P-element on the second, where 'P' refers to the P-element ($CtBP_L$, $CtBP_S$ and recombinant CtBP) driven by the 8Kb promoter region (see bottom of the figure). All chromosomes were marked with balancers to prevent recombination and distinct markers for identification of balancers. In the second step, P-element strains expressing $CtBP_L$ or $CtBP_S$ or both, were introduced into null backgrounds. The two different null alleles of CtBP were tested together to avoid possible lethality by additional accumulated lethals on the CtBP mutant chromosomes (shown as red X). In the third step, transheterozygous CtBP P-1590/1663 individuals were sought as indicated by the loss of the SM2Cyo balancer, signifying rescue from either one copy or two copies of the P-element.



Rescue with one copy of transgene

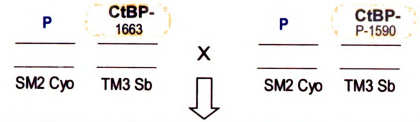
Rescue with two copies of transgene



P-element construct

Table A-1: CtBP promoter driven transgenes do not rescue CtBP mutants

Either one or two copies of transgenes were assessed for rescue in *CtBP* mutant backgrounds. Progeny were scored by genotype and categorized. All flies recovered from the final cross (Figure A-4) carried a third chromosome balancer indicting that neither $CtBP_L$, $CtBP_S$ nor both together were able to rescue lethality. Progeny carrying second chromosome P-element can be homozygous or heterozygous (with a balancer). These progeny with balancer chromosomes are generally sick and were consistently recovered at lower ratios.



Progeny	P / SM2 Cyo — CtBP-mutation / TM3 Sb	P / P — CtBP-mutation / TM3 Sb	P / SM2 Cyo — CtBP-1663 / CtBP-P-1590	P / P — CtBP-P-1590 / CtBP-1663
Expected IF NO RESCUE (CtBP mutations are homozygous lethal)	50%	50%	0%	0%
Observed-CtBPL	38	121	0	0
Observed-CtBPs	55	170	0	0
Observed-CtBPL+CtBPs	63	141	0	0

A heteroallelic CtBP mutation combination was tested to avoid mortality resulting from other uncharacterized recessive mutations that may be present on these chromosomes. All chromosomes contained identifiable markers to keep track of transgenes. Neither one nor two copies of $CtBP_L$ were able to rescue the lethality of homozygous CtBP mutations. Similar results were obtained when complementation was attempted with the short isoform alone, suggesting that maybe both forms of the protein are needed to sustain development. To test this possibility, $CtBP_L$ and $CtBP_S$ were recombined on the second chromosome and checked for rescue. However, lethality could not be rescued by simultaneous expression of both isoforms (See Table A-1).

The developmental profile of endogenous CtBP proteins shows $CtBP_S$ to be present in relatively higher amounts than the long isoform (Mani-Telang and Arnosti, 2006). However, it was noted that the expression from promoter driven transgenic lines was the opposite, with higher $CtBP_L$ levels relative to $CtBP_S$ (Figure A-2). The relative abundances of the two isoforms may be critical for proper function. A developmental profile for transgenics expressing the recombinant protein showed a detectable decrease in levels of $CtBP_L$-FLAG, mirroring the decrease in endogenous $CtBP_L$ levels, suggesting that the flag-tagged transgene is subject to the same regulation as the native proteins (Figure A-3). This decrease was more apparent with anti-CtBP antiserum (second panel) while the anti-M2 FLAG antibody did not reveal a detectable decrease; this decrease might reflect modified forms of $CtBP_L$ that do not cross-react well with the CtBP antiserum. $CtBP_L$ and $CtBP_S$ transcripts also differ in coding sequence and have different 3'UTRs. The 3'UTR is known to be important for regulating levels of protein expression either

temporally and/or spatially (Fujioka et al., 1999; Kosman and Small, 1997). It is also conceivable that we failed to include other genomic regions that carry necessary transcriptional information, for example this construct lacks a large ~3 kb intron located between exons 5 and 6 (Figure A-1) that is conserved in Drosophilids (data not shown) which might contain regulatory regions that determine expression levels of the transgene. Finally, there are at least four CtBP transcripts have been detected by northern analysis using an embryonic cDNA library that might be functionally relevant to the fly (Poortinga et al., 1998). Correspondingly, four cDNAs have been recovered (Chapter I-Figure I-4). Although the differences appear to be minor, expression of only one or two of the isoforms might not suffice in this setting to provide complete biological function.

These initial rescue experiments were attempted with cDNAs because of the ease of expressing different CtBP isoforms and mutants. An alternate approach will be to attempt rescue with the entire genomic locus contained within ~15 Kb. Such an endeavor has been successfully demonstrated for *even-skipped*, where a 16 Kb genomic region was sufficient and capable for rescuing lethality of an a null mutant (Fujioka et al., 1999). Alternative forms of the protein can then be tested in this context. A successful rescue with the full length $CtBP_L$ would set the stage for a more definitive analysis of dehydrogenase mutants and C-terminal extensions. This whole animal rescue approach has been challenging and has not been demonstrated in vertebrate models, thus these studies can add to our general understanding of CtBP in development.

MATERIALS AND METHODS

Construction of transgenes

For rescue tests, cDNA fragments for $CtBP_L$ (1.5Kb) and $CtBP_S$ (1.2Kb) were double flag-tagged at the C-termini and were inserted into the unique KpnI and XbaI sites of pCaspeR-tubulin vector. This vector additionally provides a hexahistidine fusion at the N-terminus of CtBP. For rescue using CtBP endogenous promoter (PC-CtBP), cDNA fragments for both isoforms were introduced into the KpnI and XbaI sites in the polylinker of the pUAST vector (Brand and Perrimon, 1993). The original vector has a basal hsp70 promoter and 5X UAS sites that were removed by restriction digestion with SphI and KpnI and replaced with an adaptor that containing SphI, a new AscI and KpnI restriction sites and an optimal Kozak sequence (DA 885 5'-CACCGGCGCGCCACCAAAATGGGTAC-3'; DA 886 5'-CCATTTTGGTGGCGCGCCGGTGCATG-3'). The promoter region of CtBP (~8054 bp) was amplified from embryo genomic DNA in two halves: the first 4000 bp fragment containing one of the predicted start sites was amplified as an SphI-AscI fragment (CG8841302-8845092) with DA 926 (5'-GTGCATGCGAAATGGTTAGCCAGCGTGGTG-3') and DA 927 (5'-CGGGCGCGCCTTGAAATCGAGAATCCTGCAATGG-3') and inserted immediately upstream of the cDNA. The second fragment (~4054 bp) with the other putative transcriptional start site was amplified as an SphI-SphI fragment (CG8837323-8841301) with primer sets DA 924 (5'-CTGCATGCATACCATAATTCTTGCAGTTTGCC-3') and DA 925 (5'-CGGCATGCAGCTTTCTGTTTCATGCATATGCAC -3') and introduced into

the SphI site, upstream of the first fragment. Another version of this promoter driven construct (PH-CtBP) was built to retain the basal hsp70 promoter and the 5X UAS sites, in order to achieve drive higher levels of transgene using a combination of the CtBP regulatory region and the inducible pUAS promoter but was not used in rescue. In this case, the SphI site was used to insert an SphI-AscI-SphI adaptor (DA 964 5'-CACCTCAGGCGCGCCACCTGCGCATG-3'; DA 965 5'-CGCAGGTGGCGCGCCTGAGGTGCATG-3'). The 8Kb promoter sequence was then cloned in two steps like that described for PC-CtBP.

Western Blot Analysis

Total extracts from adult flies were immunoblotted 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. Mouse monoclonal antibodies include anti-M2 used to detect FLAG epitope-tagged CtBP (1:10,000), anti-CtBP (polyclonal, 1:10,000) (Struffi and Arnosti, 2005) and β-tubulin (1:6000, Iowa Hybridoma Bank). These were visualized using HRP-conjugated secondary antibodies (Pierce) and SuperSignal® West Pico chemiluminiscent substrate (Pierce).

CtBP fly stocks

Flies carrying CtBP promoter driven transgenes were introduced into flies by injection procedures described previously (Rubin and Spradling, 1982). Transgenic lines were checked for protein expression and flies homozygous for the transgene on the second chromosome were used for rescue. To test if rescue relied on both transgenes, flies expressing PC-CtBP$_L$ and PC-CtBP$_S$ carried over balancers were crossed and allowed to recombine on the second chromosome. Progeny were screened by performing an anti-M2 western to detect both FLAG-tagged forms of CtBP$_L$ and CtBP$_S$ from single flies still carrying balancer, which is indicative of a recombined chromosome. Flies carrying a P-element insertion in CtBP (Stock #P-1590, described in (Poortinga et al., 1998) or a deletion (Stock #1663) were obtained from Bloomington Stock Center. Mutant chromosomes for CtBP were maintained over balancer chromosomes i.e. *TM3, Sb.* All genetic crosses were maintained at 25 °C on standard cornmeal/molasses medium.

REFERENCES

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-14.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Donaldson, T. D., Noureddine, M. A., Reynolds, P. J., Bradford, W. and Duronio, R. J. (2004). Targeted disruption of Drosophila Roc1b reveals functional differences in the Roc subunit of Cullin-dependent E3 ubiquitin ligases. *Mol Biol Cell* **15**, 4892-903.

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B. (1999). Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* **126**, 2527-38.

Grumbling, G., Strelets, V. and Consortium, T. F. (2006). FlyBase: anatomical data, images and queries. *Nucleic Acids Research* **34** D484-D488; doi:10.1093/nar/gkj068. http://flybase.org/

Kosman, D. and Small, S. (1997). Concentration-dependent patterning by an ectopic expression domain of the Drosophila gap gene knirps. *Development* **124**, 1343-54.

Mani-Telang, P. and Arnosti, D. N. (2006). Developmental expression and phylogenetic conservation of alternatively spliced forms of the C-terminal Binding Protein corepressor. *Development, Genes and Evolution* **In Press**.

Moran, E. and Jimenez, G. (2006). The tailless nuclear receptor acts as a dedicated repressor in the early Drosophila embryo. *Mol Cell Biol* **26**, 3446-54.

Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *Embo J* **17**, 2067-78.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science* **218**, 348-53.

Struffi, P. and Arnosti, D. N. (2005). Functional interaction between the Drosophila knirps short range transcriptional repressor and RPD3 histone deacetylase. *J Biol Chem* **280**, 40757-65.

Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol* **24**, 5953-66.

Wagner, C. R., Mahowald, A. P. and Miller, K. G. (2002). One of the two cytoplasmic actin isoforms in Drosophila is essential. *Proc Natl Acad Sci U S A* **99**, 8037-42.

APPENDIX B

AN RNAi APPROACH TO DEPLETING THE $CtBP_L$ ISOFORM

The *CtBP* gene encodes two major splice variants ($CtBP_L$ and $CtBP_S$), both of which can be detected through all developing stages of the fly (Chapter I – Figure I-4). CtBP plays vital roles during fly embryogenesis, emphasized by mutations in the gene which are homozygous lethal. It is not known whether the existence of multiple isoforms imparts functional specificity during development, however expression of single isoforms is not sufficient to rescue lethality. In other systems, there are several examples of distinctions between CtBP isoforms. Human CtBP1, but not CtBP2, is modified by sumoylation that results in its nuclear translocation. CtBP2 but not CtBP1 contains an NLS that directs it to the nucleus, suggesting that CtBP1 and CtBP2 are regulated differently. Mouse knockouts show distinct phenotypes, lending support to the hypothesis of different isoforms having specific roles.

Initial attempts for a transgene-based rescue experiment do not provide any clues regarding the functional equivalency of the two isoforms in *Drosophila* (Appendix A). Both isoforms when tethered at the promoter have been documented to display comparable repression activities (Sutrias-Grau and Arnosti, 2004). *In vivo* studies assaying biological activity of $CtBP_S$ have been of limited value due to the low expression levels of this protein. However, we observe a decline in $CtBP_L$ protein levels from the larval to adult stages (Mani-Telang and Arnosti, 2006). Whether this translates to $CtBP_S$ supplying $CtBP_L$ like functions or whether $CtBP_L$ is no longer required during these developmental stages and is hence down regulated, remains to be

determined. This downregulation might transpire by post-translational modifications that occur within the C-terminal tail (Chinnadurai, 2006) (Also see Chapter 1).

As an independent yet complementary approach, we tried to selectively deplete levels of $CtBP_L$ isoform to gain insights into isoform-specific roles. To inhibit $CtBP_L$ levels, we used RNA interference (RNAi) in combination with the inducible UAS-Gal4 system in a fly model. RNAi by double stranded RNA was first described in worms and the mechanism has since been found to be conserved in vertebrates, invertebrates and plants (Reichhart et al., 2002). In *Drosophila*, the UAS-Gal4 system has been successfully applied to study gain-of-function and more recently, loss-of-function phenotypes; the latter resulting from gene specific perturbations by RNAi (Duffy, 2002; Roman, 2004). In *Drosophila*, The C-terminal region of $CtBP_L$ is absent in $CtBP_S$ (includes exon 6+7 and a ~700 bp 3'UTR) and represents a unique sequence by which this particular isoform can be targeted. A responder line consisting of tandem inverted repeats to the C-terminal tail region of $CtBP_L$ (See Materials and Methods) were cloned downstream of the UAS sites in pUAST and were expressed ubiquitously using a constitutive Actin5C-Gal4 driver line (See Figure B-1). This cross tests the efficacy of the hairpins to knockdown $CtBP_L$ by inducing the expression of hairpins in a ubiquitous fashion early in development. If the function of $CtBP_L$ is mandatory during these times, then lethality among progeny is expected. If lethality were to be observed, it would validate that interference with CtBP function is possible, following which the next step would be test the specificity of knockdown. RNAi has been demonstrated to be gene specific, but it is uncertain if the inhibitory effects

can spread to related family members (like splice variants). It is possible that the inverted repeats will also cause a depletion of $CtBP_S$ levels. This possibility will be tested by crossing the responder line to a tissue specific driver, and testing CtBP levels in this tissue by a western blot. These experiments are currently in progress.

If specific knockdown of $CtBP_L$ levels are observed, I shall analyze outcomes arising from depletion of this isoform at various stages and in different tissues during development. Another possibility is that partial knockdown of $CtBP_L$ is observed, in which case it would still be interesting to analyze phenotypes from temporal and spatial reductions of the long isoform.

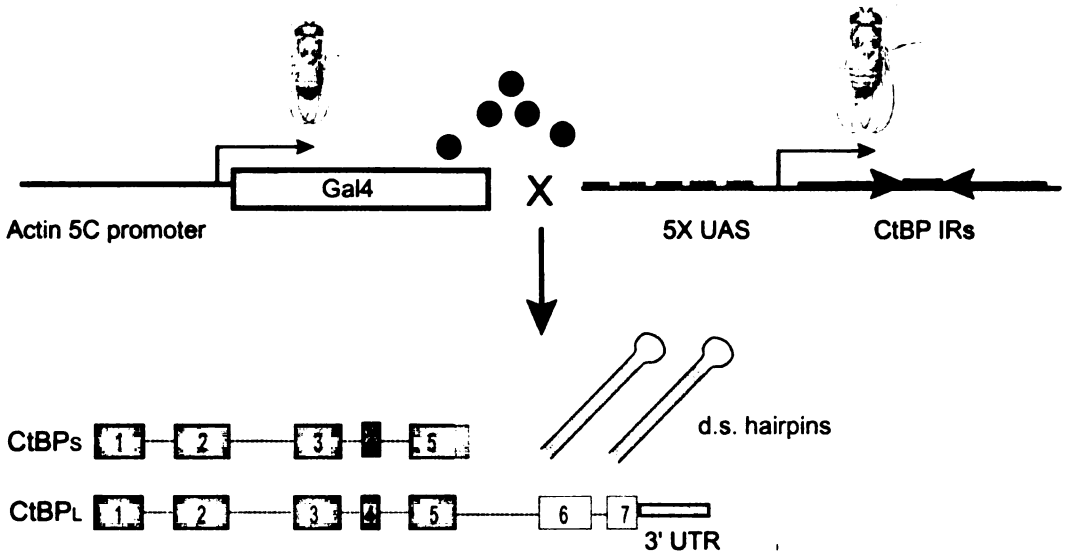


Figure B-1: A transgenic system to trigger RNAi and selectively eliminate $CtBP_L$ in *Drosophila*

A transgenic driver line expressing Gal4 activator under the control of a ubiquitous driver (Actin 5C) is crossed with a transgenic responder line expressing tandem inverted repeats against $CtBP_L$, downstream of UAS-regulatory sites. The responder line was made homozygous for the P-element carrying the inverted repeats and the driver line is maintained over a balanced chromosome. The results of $CtBP_L$ knockdown will be judged by screening progeny lacking the balancer-marker resulting from this cross.

MATERIALS AND METHODS

Construction of transgenes

Tandem inverted repeats were cloned into the pUAST vector that allows expression of dsRNA hairpins *in vivo*, facilitating inducible knockdown of $CtBP_L$ (Brand and Perrimon, 1993). Two types of double stranded hairpin constructs were generated. The first construct was designed to target coding sequence for exons 6+7 in $CtBP_L$ (221 bp+81 bp at the C-termini). Inverted repeats (IRs) of this ~300 bp sequence were amplified using $CtBP_L$ cDNA as a BglII-NotI fragment using primer pairs DA 1219 (5'-CTGGATAGATCTGCACTGCATCACCGGGCACAC-3') and DA1220 (5'-ATAAGATGCGGCCGCCGGCGCCTCCGTTGACTC-3') for the forward repeat .The reverse repeat was amplified from as a KpnI-XhoI fragment using DA 1221 (5'-GATGGTACCGCACTGCATCACCGGGCACAC-3') and DA 1222 (5'-GATCTCGAGCGGCGCCTCCGTTGACTCGG-3') from $CtBP_L$ cDNA. The second construct was designed to target unique 3' exon regions encoding $CtBP_L$ 'tail', along with ~700 bp of 3' UTR sequence specific to this isoform. Inverted repeat sequences (~1.3 Kb) were amplified as a BglII-NotI fragment using primer pairs DA 1219 (see above) and DA 1223 (5'-ATAAGATCAGCGGCCGCGTTTCTCGTAATTAAAATTTTCCAAC-3') for the forward repeat and primer pairs DA 1261 (5'-TATTCTAGAGCACTGCATCACCGGGCAC-3') and DA 1262 (5'-TCAGGTACCGTTTCTCGTAATTAAAATTTTCCAAC-3') for the reverse repeat amplified as a XbaI-KpnI insert. In both constructs, the inverted repeats were separated from each other using an ~800 bp region containing the

second intron of the *mub* transcript (CG7437) amplified with primers DA 753 (5'-ATAAGATCAGCGGCCGCCAGGACGTCCAATCAAAGTGGTCAAACCCG-3') and DA 754 (5'-CTGGATCTCGAGGGCTGGAGTTCAATAAATATACCATCGCTCTTTGGC-3') (Used in Reichhart et al., 2002).

Fly Stocks and Expression System

Transgenic fly lines carrying the UAS-RNAi vectors were obtained using standard techniques of P-element mediated germline transformation. Multiple independent lines for each construct were made homozygous and all crosses were performed at 25°C. Knockdown efficacy was tested by crossing to a strong ubiquitous actin5C-Gal4 driver line (Bloomington Stock Center, Stock # 4414) that is carried over a marked balancer chromosome. The progeny of this cross will first be screened for any flies that carry non-marked chromosomes and if such progeny emerge, will be validated for CtBP protein levels by western blotting with an anti-CtBP antisera (as described in Chapter II- Materials and Methods).

REFERENCES

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Chinnadurai, G. (2006). CtBP family proteins:unique transcriptional regulators in the nucleus with diverse cytosolic functions: Landes Bioscience.
Duffy, J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis* **34**, 1-15.

Mani-Telang, P. and Arnosti, D. N. (2006). Developmental expression and phylogenetic conservation of alternatively spliced forms of the C-terminal Binding Protein corepressor. *Development, Genes and Evolution* **In Press**.

Reichhart, J. M., Ligoxygakis, P., Naitza, S., Woerfel, G., Imler, J. L. and Gubb, D. (2002). Splice-activated UAS hairpin vector gives complete RNAi knockout of single or double target transcripts in Drosophila melanogaster. *Genesis* **34**, 160-4.

Roman, G. (2004). The genetics of Drosophila transgenics. *Bioessays* **26**, 1243-53.

Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Mol Cell Biol* **24**, 5953-66.