STRUCTURAL-FUNCTIONAL ANALYSIS OF THE TRANSCRIPTIONAL COREPRESSOR C-TERMINAL BINDING PROTEIN IN *DROSOPHILA*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Biochemistry and Molecular Biology

2011

ABSTRACT

STRUCTURAL-FUNCTIONAL ANALYSIS OF THE TRANSCRIPTIONAL COREPRESSOR C-TERMINAL BINDING PROTEIN IN *DROSOPHILA*

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Transcriptional cofactors play complex roles in developmental gene regulation. The evolutionarily conserved transcriptional corepressor C-terminal binding protein (CtBP) is recruited by a variety of transcriptional factors that play crucial roles in development and disease; this protein contains a central NAD(H)-binding core domain homologous to D2 hydroxy acid dehydrogenase enzymes, as well as an unstructured C-terminal domain. NAD(H) binding is important for CtBP repression activity and enables CtBP to function as a metabolic sensor to regulate gene expression through cellular metabolic status. The striking homology of CtBP to metabolic dehydrogenase enzymes and the presence of an enzymatic activity in CtBP also raise the possibility of a direct link between cellular metabolism and transcriptional regulation. The unstructured C-terminus is susceptible to modifications by sumoylation and phosphorylation, which can influence CtBP subcellular localization and stability.

In this study, we established genetic rescue assays to determine how the enzymatic activity and the C-terminal domain of CtBP function in the context of *Drosophila melanogaster* development. The mutant phenotypes and specific gene regulatory effects indicate that both the catalytic site of CtBP and the C-terminal domain play important roles in development. Our results indicate that the structural and enzymatic features of CtBP, previously thought to be dispensable for overall transcriptional control, are critical for modulating this protein's activity in diverse developmental settings.

In addition, we identified hundreds of potential CtBP target genes affected by the enzymatic activity and the C-terminal domain of CtBP by genome-wide transcriptome analysis. We show that these enzymatic and structural features regulate multiple cellular processes such as metamorphosis and metabolism. Particularly, we show that CtBP may regulate *Drosophila* innate immunity, a potential novel function implied by CtBP. Taken together, our results may open doors to discover novel functions of CtBP in multiple biological processes.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance of my advisor and committee members, help from friends, and support from my family.

My deepest gratitude is to my advisor, Dr. David Arnosti. It has been an honor to be one of his Ph.D. students, and a good fortune to work with him. His profound knowledge, enthusiasm for research, and critical thinking set a perfect example of an excellent scientist. I greatly appreciate all his contributions of time and efforts to teach me to design experiments properly, to discuss papers for prelim, and to discuss numerous problems encountered during this Ph.D. project. It is his excellent guidance that brings me to the scientific world. I am also thankful for his understanding and support throughout the Ph.D. pursuit.

I would like to thank my committee members, Dr. Lee Kroos, Dr. William Henry, Dr. Alexander Shingleton, and Dr. Zachary Burton, for their critical suggestions and insightful comments. I am deeply grateful to Dr. Lee Kroos and Dr. William Henry for their great support of my postdoctoral position hunting. Special thanks go to Dr. Zachary Burton who was willing to serve on my committee at the last moment.

I would like to thank the members of the Arnosti lab who have been good friends and colleagues in the past few years. I am especially grateful to Dr. Sandhya Payankaulam, Dr. Priya Mani-Telang, and Dr. Li Li for their insightful suggestions. I also thank Rupinder Sayal, Pankaj Acharya, Liang Zhang, and Jackie Dresch who has been very helpful on bioinformatics and confocal microscopy. It is my pleasure to work with them and I greatly value their friendship.

I would like to thank my family for their love and support. I thank my parents and sister for their love and unwavering faith in me. I am also indebted to my parents-in-law for their support,

especially my mother-in-low who has taken very good care of my family when I was not able to. Finally and most importantly, I owe my deepest gratitude to my wife, Dr. Zhihong Wang, who has been tremendously supportive in the last three years. I am greatly grateful for her love, understanding, encouragement, and patience, which have made this dissertation possible. I also would like to thank my beloved daughter, Sophia Zhang, who has brought me the greatest joy and made the life more meaningful.

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LIST OF ABBREVIATIONS

2DG	2-deoxy-D-glucose
AMPs	antimicrobial peptides
AN	Angustifolia
APC	adenomatous polyposis coli
ARF	alternative-reading-frame protein
BRCA1	breast cancer 1
CBP	CREB binding protein
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CoREST	corepressor of REST
CREB	cAMP response element-binding protein
CtBP	C-terminal binding protein
DAVID	the database for annotation, visualization and integrated discovery
DNA	Deoxyribonucleic acid
EMT	epithelial-mesenchymal transitions
EST	expressed sequence tag
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
HAT	histone acetyltransferase
HDAC	Histone deacetylase
HIC1	hypermethylated in cancer 1

HIPK2	homeodomain interacting protein kinase 2
HMT	histone methyltransferase
Imd	Immune deficiency
LPAAT	lysophosphatidic acyl transferase activity
LSD1	Lysine specific demethylase 1
MEF	mouse embryonic fibroblasts
mRNA	messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NLS	nuclear localization signal
NRSF	neural restrictive silencing factor
PAK1	p21-activated kinase 1
PCAF	p300/CBP-associated factor
PGRP	peptidoglycan recognition protein
REST	RE1 silencing transcription factor
SEM	scanning electron microscopy
siRNA	small interfering RNA
SIRT1	sirtuin 1
SUMO	small ubiquitin-related modifier
TCF	T cell factor
THAP	Thanatos-associated protein
TSA	trichostatin A
TSS	transcription start site

CHAPTER 1

INTRODUCTION

Transcriptional cofactors play complex roles in developmental gene regulation. The conserved transcriptional corepressor CtBP was initially identified in mammalian cells through its association with the C-terminus of the adenovirus E1A oncoprotein (Boyd et al., 1993; Schaeper et al., 1995) and was later characterized as a transcriptional corepressor that interacts with and mediates repression activity of short-range transcriptional repressors in *Drosophila* (Nibu et al., 1998b; Poortinga et al., 1998). The essential role of CtBP in animal development has been definitively illustrated by genetic studies in both *Drosophila* and mice, where loss of the CtBP function causes embryonic lethality (Hildebrand and Soriano, 2002; Poortinga et al., 1998). Subsequent studies revealed that CtBP is recruited to a wide variety of transcription factors to mediate their transcriptional activity (Chinnadurai, 2002; Turner and Crossley, 2001). CtBP has also been suggested to play a role in non-transcription-related processes, namely Golgi membrane fission (Weigert et al., 1999) and synapse formation (Schmitz et al., 2000). In addition, CtBP also plays important roles in oncogenesis (Chinnadurai, 2002, 2009). CtBP has been shown to suppress E1A-mediated transformation (Boyd et al., 1993; Schaeper et al., 1995), promote cancer cell migration (Zhang et al., 2006), and repress multiple tumor suppressors (Chinnadurai, 2009).

CtBP is also an NAD-dependent dehydrogenase. It shows striking sequence similarities to NAD(H)-dependent D-2-hydroxyacid dehydrogenases (Schaeper et al., 1995) and contains a highly conserved NAD(H)-binding motif (Rossmann fold) and a putative His-Glu-Arg triad active site, where the histidine plays an essential role in catalysis (Chinnadurai, 2002; Kumar et al., 2002). These two structural features are absolutely conserved in all animal CtBP proteins

studied, suggesting they might be functionally significant. Numerous studies have demonstrated that NAD(H) binding is essential for CtBP function (Kumar et al., 2002; Kuppuswamy et al., 2008; Nardini et al., 2009; Sutrias-Grau and Arnosti, 2004; Thio et al., 2004; Zhang et al., 2002), but the biological relevance of the putative enzymatic activity remains elusive.

Mechanistically, CtBP acts as a corepressor to repress target gene expression by altering chromatin structure. As discussed below, CtBP forms a complex with multiple histone modification enzymes, including histone deacetylases HADC1/2, histone methyltransferases, and the histone demethylase, LSD1 (Shi et al., 2004; Shi et al., 2003). Additionly, CtBP may repress gene expression by antagonizing transcriptional coactivator activities (Chinnadurai, 2007).

1.1 CtBP family proteins

CtBP is a highly conserved transcriptional corepressor found in multicellular eukaryotes. The human CtBP1 was first identified in the Chinnadurai laboratory as a 48 kD phosphoprotein that associated with the C terminus of adenovirus E1A protein. The cDNA for CtBP was cloned two years later using the yeast two-hybrid system (Boyd et al., 1993; Schaeper et al., 1995). An EST database screen using *CtBP1* cDNA sequences identified a second *CtBP* gene, *CtBP2*, in human and mouse (Katsanis and Fisher, 1998). The *CtBP1* gene has been mapped to chromosome 4 and chromosome 5 in human and mouse, respectively, while the *CtBP2* is located on chromosome 10 of human and chromosome 7 of mouse (Chinnadurai, 2006). Invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* possess a single *CtBP* gene (Poortinga et al., 1998). Other *Drosophila* species and more distantly related insects such as *Anopheles gambiae*, and *Apis mellifera*, also contain a single *CtBP* gene with multiple splice forms (Mani-Telang and Arnosti, 2007). A *CtBP* homolog *ANGUSTIFOLIA* (*AN*) has also been identified in *Arabidopsis*

thaliana; this gene has a role in regulating leaf-cell expansion (Folkers et al., 2002; Kim et al., 2002). The connection with transcriptional regulation by *AN* is unclear; the protein lacks characteristic structural features and forms a distinct phylogenetic subgroup, suggesting it may function differently from animal CtBP proteins. Indeed, functional analysis showed that *AN* was not able to interact with the E1A protein, failed to repress reporter gene expression, and cannot rescue the *CtBP*-null phenotype in *Drosophila* (Stern et al., 2007).

Surprisingly and interestingly, CtBP is also a NAD(H)-dependent dehydrogenase. It shows striking sequence and structural similarities to D-2-hydroxyacid dehydrogenases (Kumar et al., 2002; Schaeper et al., 1995). Similar to its bacterial counterparts, CtBP contains a highly conserved NAD(H)-binding motif (Rossmann fold) and a putative His-Glu-Arg triad active site, where the histidine plays an essential role in catalysis (Chinnadurai, 2002; Kumar et al., 2002). Biochemical studies have shown that CtBP is an bona fide NAD(H)-dependent dehydrogenase that exhibits weak activity *in vitro*, reducing pyruvate to lactate with concomitant oxidation of NADH to NAD^+ (Kumar et al., 2002). However, a physiological *in vivo* substrate has not been identified, and it has been unclear whether the active site residues are important for transcriptional function, as mutant forms of CtBP lacking these conserved amino acids can still mediate repression (Grooteclaes et al., 2003; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004). The NAD(H)-binding activity is however critical for CtBP repression activity. Disrupting NAD(H)-binding affects CtBP dimerization (Kuppuswamy et al., 2008; Nardini et al., 2009; Thio et al., 2004), association with transcription factors (Kumar et al., 2002; Zhang et al., 2002), cellular localization (Kuppuswamy et al., 2008; Zhang and Arnosti, 2011), and abolishes its repression activity (Sutrias-Grau and Arnosti, 2004; Zhang and Arnosti, 2011). In addition, CtBP has been reported to preferentially bind to NADH over NAD^+ with >100-fold higher affinity

(Fjeld et al., 2003; Nardini et al., 2003; Zhang et al., 2002). This selectivity has been hypothesized to permit CtBP to respond to changes in the balance of these two forms of dinucleotide, positioning this corepressor as a possible sensor of cellular redox status. Consistent with this idea, changing the cellular NADH/ NAD^+ ratio has profound effects on CtBP-mediated target gene repression (Kim et al., 2005; Zhang et al., 2002; Zhang et al., 2006; Zhang et al., 2007).

CtBP genes produce multiple isoforms through alternative RNA splicing and promoter usage. In mammals, two splice isoforms, CtBP1-L and CtBP1-S (also known as CtBP3/BARS) are produced from the *CtBP1* gene. The CtBP2 and RIBEYE transcripts are produced from the *CtBP2* locus by alternative promoter usage (Chinnadurai, 2006; Schmitz et al., 2000). CtBP1-L was the first CtBP family protein to be identified; this protein functions as a transcriptional corepressor. CtBP1-S, however, has been suggested to possess a lysophosphatidic acyl transferase activity (LPAAT) and induce fission of Golgi membranes during mitosis (Weigert et al., 1999). However, more recent studies by Gallop et al. showed that CtBP1-S itself did not have LPAAT activity; this enzymatic activity may instead co-purify with CtBP1-S (Gallop et al., 2005). The CtBP2 isoform is structurally similar to CtBP1-L and also functions as a corepressor. The RIBEYE protein, however, contains a unique N-terminal A domain (Schmitz et al., 2000). This protein is a major component of synaptic ribbons, a specialized structure in certain vertebrate neurons, and the A domain may mediate aggregation of RIBEYE in ribbons.

Alternative splice forms of CtBP are also found in invertebrates. In *Drosophila*, the single CtBP gene encodes at least three splicing isoforms containing 383, 386, and 479 amino acids respectively (Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998). Northern blot analysis revealed three major CtBP mRNA species with sizes about 2.5, 2.7, and 4.0 kb

expressed throughout *Drosophila* development. The longer isoform of CtBP contains a nonconserved C-terminal domain which is not included in the short isoform. The 479-amino-acid version is designated as $CtBP_L$ and the 383-amino-acid isoform as $CtBP_S$ in our lab (Sutrias-Grau and Arnosti, 2004). Both the $CtBP_L$ and $CtBP_S$ are expressed through *Drosophila* development and are conserved in insects (Mani-Telang and Arnosti, 2007). The C-terminal extension present in the *Drosophila* $CtBP_L$ isoform is a feature shared with other metazoan CtBP proteins, and multiple posttranslational modifications have been shown to occur in this region (Lin et al., 2003; Zhang et al., 2005; Zhang et al., 2003), indicating it may play regulatory roles. The *Drosophila* $CtBP_S$ isoform, which is the predominant form in the fly, does not have this domain. Although $CtBP_S$ and $CtBP_L$ are co-expressed throughout development, their relative abundances vary in an evolutionarily conserved fashion, suggesting these two isoforms may have distinct functions (Mani-Telang and Arnosti, 2007).

1.2 Structural features of CtBP

The CtBP primary structure can be divided into three functional domains: the N-terminal substrate binding domain, the conserved dehydrogenase domain, and the C-terminal extension (Figure 1). The substrate binding domain and dehydrogenase domain are conserved and form separate tertiary structural domains which are linked by a flexible hinge (Kumar et al., 2002; Nardini et al., 2003). The cleft surrounded by these two domains contains NAD(H) binding sites. The C-terminal extension is very flexible and intrinsically unstructured (Nardini et al., 2006).

The substrate binding domain mediates association of CtBP with transcription factors. A stretch of amino acids between the dehydrogenase domain and the C-terminal extension (327 -

352 of human CtBP1-L) is also part of the substrate binding domain as revealed by the crystal structures (Kumar et al., 2002; Nardini et al., 2003). This domain specifically recognizes the PXDLS consensus sequence located on binding partners through a binding surface cleft in the N-terminal part (Figure 1B). Following the identification of E1A as the first CtBP interacting transcription factors, a wide variety of transcription factors containing the CtBP interacting PXDLS motif have been found (Chinnadurai, 2002; Turner and Crossley, 2001), including the short-range repressors Knirps (Kni), Kruppel (Kr), Snail (Sna), and Giant (gt) that are critical for *Drosophila* early patterning (Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998). It was the identification of CtBP as a crucial interacting partner of these short-range repressors that defined CtBP as a transcriptional corepressor (Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998). Due to the diversity of the transcription factors that interact with CtBP, CtBP has been suggested to play roles in various cellular processes. For example, the associations of CtBP with the adenomatous polyposis coli (APC) protein (Hamada and Bienz, 2004), a component of the destruction complex promoting degradation of β-catenin, and the *Xenopus* Tcf-3 protein (Brannon et al., 1999) reveal roles of CtBP in the Wnt signaling pathway; while the E1A-CtBP interaction and numerous tumor suppressors repressed by CtBP (Chinnadurai, 2009) definitely speak of the importance of CtBP in oncogenesis.

The N-terminal domain also mediates interaction with transcriptional co-regulators, including histone deacetylases. The class III HDACs, HDAC4, -5, and -7 have been shown to contain the conserved CtBP interaction motif in the N-terminal region, and a direct association between CtBP and HDAC7 has been revealed by GST pull-down assays (Dressel et al., 2001; Turner and Crossley, 2001; Zhang et al., 2001). Although other HDACs such as HDAC1/2 do not contain the PXDLS consensus, they also copurify with the mammalian CtBP complex (Shi et al., 2003).

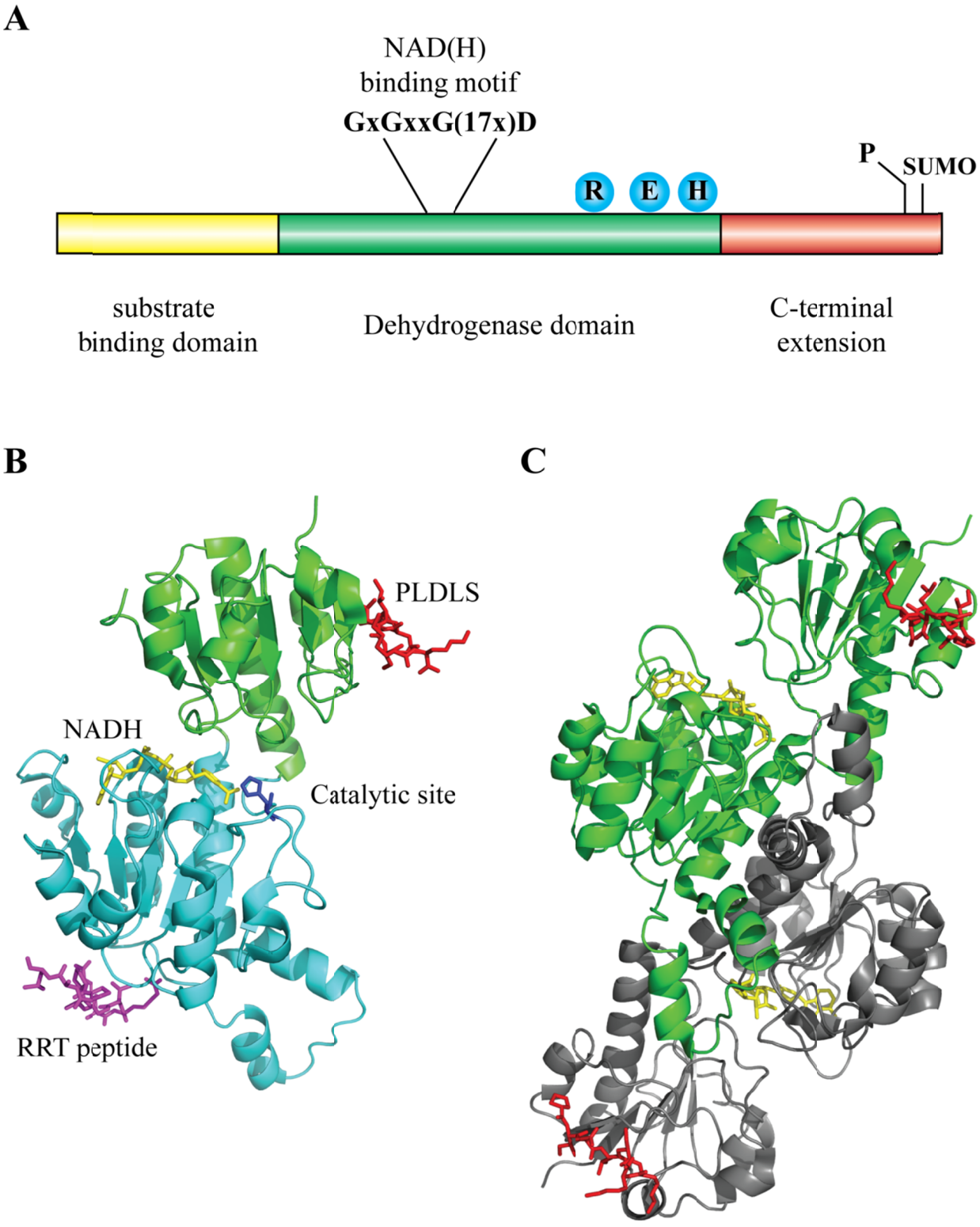

Figure 1. CtBP structure.

Figure 1 (cont'd)

Figure 1. CtBP structure. (A) Domain structure of mammalian CtBP protein. The N-terminal substrate binding domain, central dehydrogenase domain, and C-terminal domain are highlighted in yellow, green, and red respectively. The conserved NAD(H) binding motif is shown. The catalytic triad His-Glu-Arg residues are depicted as blue circle. P and SUMO represent phosphorylation and sumoylation site, respectively. (B) Crystal structure of CtBP. The substrate binding and dehydrogenase domain are colored in green and cyan respectively. The bound NADH, PLDSL and RRT peptide are highlighted in yellow red and purple, respectively. The putative catalytic histidine residue is highlighted in blue. (C) Dimeric structure of CtBP. The dimerization is mainly mediated by the dehydrogenase domain, leaving the substrate domain of each monomer at two extremes. The two CtBP monomers are shown in green and gray, respectively. Bound PLDLS peptide and NAD(H) are highlighted in red and yellow. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Mutating key residues in this binding cleft abolishes not only interactions with transcription factors, such as E1A, but also chromatin modifiers such as HDACs, histone demethylase LSD1, histone methyltransferases G9a, and corepressor of REST (CoREST) - components of the CtBP complex - indicating that the PXDLS binding cleft region may serve as the primary center for recruitment of sequence-specific transcription factors and enzymatic constituents of the corepressor complex (Kuppuswamy et al., 2008). CtBP also contains a second groove that binds to conserved RRTGXPPXL sequences (RRT motif) (Quinlan et al., 2006). This RRT motif has been found in zinc finger proteins such as ZNF217, RIZ, and ZNF516. This RRT binding groove is functionally redundant with the PXDLS binding cleft, and may be important for interacting with specific proteins, such as ZNF217 and G9a, but not all CtBP interacting partners (Kuppuswamy et al., 2008; Quinlan et al., 2006). The RRT binding groove has been mapped to the NAD(H) binding domain and is separated from the PXDLS binding cleft. However, no apparent conformational changes in CtBP have been observed along with binding of either the PXDLS or the RRT consensus peptide (Nardini et al., 2003; Quinlan et al., 2006).

A nuclear localization signal (NLS) has also been found in this N-terminal region. Verger et al. noticed that the mammalian CtBP2 and the *Drosophila* CtBP proteins contain a potential NLS signal KRQR in the N-terminus; deleting the N-terminal region or mutating the putative NLS resulted in localization of CtBP to the cytoplasm, demonstrating an essential role of this NLS in CtBP cellular localization (Verger et al., 2006). Surprisingly, this NLS can be post-translationally modified (Zhao et al., 2006). Acetylation of the lysine residue in this NLS by p300 plays important roles in CtBP nuclear retention (Zhao et al., 2006).

The second major structural feature of CtBP proteins encompasses the NAD(H) binding domain, which shows striking homology to NAD(H)-dependent D-2-hydroxyacid

dehydrogenases. This similarity was immediately noticed upon the cloning of the *CtBP1* cDNA, indicating CtBP might be a functional dehydrogenase, but initial attempts to evaluate NAD(H) binding or the enzymatic activity were unsuccessful (Schaeper et al., 1995). Kumar and colleagues, however, reported in 2002 that CtBP does possess a bona fide dehydrogenase activity (Kumar et al., 2002). They showed that the purified CtBP protein can catalyze, though at slower rate, the reduction of pyruvate to lactic acid in a dose-dependent manner, while a catalytic site mutant failed to do so. They crystallized CtBP in the presence of NAD^+ and showed in the crystal structure that CtBP forms a complex with NAD^+, which was independently confirmed by other labs (Nardini et al., 2003; Quinlan et al., 2006).

Similar to bacterial dehydrogenases, the CtBP NAD(H) binding domain contains a highly conserved NAD(H)-binding motif, the GXGXXG(17X)D Rossmann fold, and a putative active site, the conserved His-Glu-Arg triad, in which the histidine is essential for catalytic activity (Figure 1) (Chinnadurai, 2002; Kumar et al., 2002). The presence of an NAD(H)-dependent dehydrogenase activity in CtBP, a transcriptional corepressor, potentially adds dehydrogenases to the list of transcriptional regulatory enzymes, such as histone acetyltransferases, deacetylases, methyltransferases and demethylases, and raises the interesting question that it may directly bridge cellular metabolism and gene regulation. Kumar and coworkers showed that a series of mutations affecting the putative catalytic site of CtBP compromised physical interaction with E1A protein *in vitro* and repression activity *in vivo* (Kumar et al., 2002), however, the importance of this putative activity in gene regulation is controversial. First, the "catalytic" mutant tested by Kumar also disrupted an arginine thought to be involved in NADH binding. Second, more subtle mutations that affect only the critical histidine residue necessary for catalytic activity, confirmed using *in vitro* assays, do not disturb *in vivo* repression activity

(Balasubramanian et al., 2003; Kumar et al., 2002; Phippen et al., 2000; Sutrias-Grau and Arnosti, 2004; Turner and Crossley, 1998). This enzymatic activity has also been suggested to be dispensable when tested in developmental contexts. For example, overexpression of a catalytic mutant form of CtBP induced eye and wing phenotypes in *Drosophila* similar to those produced by overexpression of the wild type protein (Mani-Telang et al., 2007). In addition, loss of *CtBP* function in *C. elegans* produces an extended life span phenotype, which is fully suppressed by either the wild type or the dehydrogenase-defective CtBP (Chen et al., 2009). A genome-wide transcriptome analysis comparing CtBP targets expressed in CtBP wild type- and catalytic mutant-rescued mouse embryo fibroblast cells also suggested that the enzymatic activity is not required (Grooteclaes et al., 2003). One possible explanation of why Kumar et al. observed dramatic effects of the catalytic activity in their report might be that multiple residues along with the critical histidine site have been mutated for that particular catalytic mutant (Kumar et al., 2002).

To complicate this issue further, Phippen et al. reported that CtBP's enzymatic activity is important for the activation function of CtBP (Phippen et al., 2000). Although CtBP functions mainly as a transcriptional corepressor, Phippen and colleagues showed that CtBP activates reporter gene expression in a cell type specific manner; expression of *Drosophila* CtBP fused to Gal4DBD activated a UAS reporter in 293 and B78 cells but strongly repressed the same reporter in NIH 3T3 and CV-1 cells. Interestingly, disrupting the catalytic activity abrogated CtBP's activation potential while it had no effect on repression. The authors also proposed that CtBP interferes with repression function of Sin3, Mad, and Gro.

Unlike the enigmatic enzymatic activity, NAD binding has been demonstrated to be crucial for CtBP function. CtBP acts as a dimer; although the monomeric CtBP can bind transcription

factors and histone modification enzymes, it fails to silence target gene expression (Kuppuswamy et al., 2008). The majority of the dimerization interface resides in the NAD(H) binding domain and covers ~ 3368 $Å^2$ on each monomer (Kumar et al., 2002; Nardini et al., 2003). Structural studies reveal that NAD(H) binding induces a conformational change and brings the substrate and NAD(H) binding domains in close contact, referred to as the "closed" state (Kumar et al., 2002; Nardini et al., 2003). This NAD(H) binding induced closed state is required to stabilize tight protein dimerization. In line with the structural perspectives, biochemical studies showed that NAD(H) binding greatly stimulated CtBP oligomerization; on the other hand, diminishing NAD(H) binding by mutating key residues in the NAD(H) binding Rossmann fold severely destabilized CtBP dimerization, although basal levels of dimer can still be observed (Balasubramanian et al., 2003; Nardini et al., 2009; Thio et al., 2004).

NAD(H) binding also impacts CtBP's ability to associate with transcription factors. Similar to that NAD(H) stimulates CtBP dimerization, the presence of NAD(H) facilitates CtBP interaction with the E1A protein and the ZEB transcription repressor *in vitro* and *in vivo*. A NAD(H) binding mutant was not stimulated by addition of NAD(H), showing only basal interaction levels (Balasubramanian et al., 2003; Kumar et al., 2002; Zhang et al., 2002). Although both NAD^+ and NADH can facilitate protein interactions by CtBP, the reduced form of the dinucleotide has been suggested to be two to three orders of magnitude more effective (Fjeld et al., 2003; Zhang et al., 2002). This observation led Goodman and colleagues to propose that CtBP might function as a cellular redox sensor and the repressor function be regulated by nuclear NADH. To test this idea, they altered cellular redox status (NAD^+/NADH ratio) by treating cells with agents such as $CoCl_2$, NaN_3, or inducing hypoxia, treatments that can increase

nuclear NADH levels, and observed a significant increase of ZEB:CtBP interaction and enhanced repression activity (Zhang et al., 2002). They further showed that a similar regulatory mechanism occurs *in vivo* at endogenous targets and plays role in tumorigenesis. Zhang and coworkers found that increased NADH levels in cancer cells caused by hypoxia promoted recruitment of CtBP to the E-cadherin promoter and enhanced E-cadherin gene silencing, effects that were further correlated with increased tumor cell migration (Zhang et al., 2006). In addition, they found that the regulation of SIRT1 expression by the HIC:CtBP complex is also modulated by metabolism through the redox sensor ability of CtBP (Zhang et al., 2007). By treating cells with glycolytic inhibitor 2-deoxy-D-glucose (2DG), which blocks glycolysis and decreases cellular NADH levels, Zhang et al. observed a reduced physical interaction between CtBP and HIC1, as well as a deceased recruitment of CtBP to the SIRT1 promoter, accompanied by increased SIRT1 expression. However, NADH does not always enhance repression activity. Garriga-Canut et al. reported that increased NADH levels reduced CtBP mediated repression function (Garriga-Canut et al., 2006). They found that CtBP interacts with the neural restrictive silencing factor (NRSF), a master transcription factor in neurogenesis (Chong et al., 1995; Schoenherr and Anderson, 1995), and is required for NRSF-mediated repression of target genes BDNF and TrkB. This repression, however, is suppressed by $CoCl_2$ or hypoxia treatment, which increases cellular NADH levels, but enhanced by citrate or 2DG treatment, which decreases nuclear NADH level. 2DG reduces epilepsy progression in the rat "kindling" model, which may be through changes in NRSF:CtBP dependent metabolic regulation of target genes (Garriga-Canut et al., 2006). Interestingly, Kim and colleagues showed that CtBP antagonizes p300-mediated transcriptional activation by direct association with its bromodomain (Kim et al., 2005). NADH, however, weakens the p300:CtBP interaction, and abolishes endogenous p300:CtBP

when added directly to cells. Taken together, although NADH binding profoundly influences interaction of CtBP with transcription factor and repression outcome, it likely works in a context-dependent manner.

Beside affecting CtBP dimerization and interaction with transcription factors, NAD(H) binding may also regulate protein cellular localization. A CtBP NAD(H) binding mutant with double glycine mutation in the NAD(H) binding Rossmann fold showed inappropriate nuclear localization in MEF90 ($CtBP^{-/-}$) cells (Kuppuswamy et al., 2008). Our very recent study also revealed that a CtBP NAD(H) binding mutant localizes largely in cytoplasm in the absence of endogenous CtBP proteins (Zhang and Arnosti, 2011), indicating that NAD(H) binding may also regulate CtBP function through modulating protein localization. *Drosophila* CtBP and mammalian CtBP2 contain nuclear localization signals (NLS) in the N-termini. The CtBP1 protein lacks this motif; nuclear localization of CtBP1 largely relies on heterodimerization with CtBP2 (Verger et al., 2006). In light of this indirect method for nuclear targeting, it is conceivable that NADH may affect CtBP nuclear localization by stimulating CtBP dimerization and interaction with transcription factors.

Although the structure and function of the dehydrogenase-like central portion of CtBP has been carefully studied, the functional significance of the C-terminal domain, is not well understood. Although this region is present in all mammalian CtBP proteins and the $CtBP_L$ isoform in *Drosophila*, it is less conserved than the central core (Nardini et al., 2006). The C-terminal region is rich in glycines and prolines, contains little predicted secondary structure, and is intrinsically unstructured. In fact, all the CtBP crystal structures available so far lack this region (Kumar et al., 2002; Nardini et al., 2003). In addition, in certain assays, this C-terminal extension seems dispensable for CtBP repression activity. $CtBP_S$, the naturally present isoform

of CtBP in *Drosophila*, does not contain the C-terminal domain but possesses similar repression activity when tethered directly to DNA (Sutrias-Grau and Arnosti, 2004). Interestingly, the $CtBP_S$ isoform is the most abundant form in *Drosophila*, suggesting the C-terminal extension might not play a major role in CtBP function (Mani-Telang and Arnosti, 2007). Similarly, the dehydrogenase core region of human CtBP1 lacking the C-terminal extension showed wild-type interaction with a transcription factor and repression activity on a reporter gene (Kumar et al., 2002).

The C-terminal region may not be crucial for CtBP function, but studies revealed that it might play regulatory roles. Lin et al. and Kagey et al. showed independently that human CtBP1, but not CtBP2, contains a single putative SUMO motif (427-VKPE-430) in the distal C terminus and provided strong biochemical evidence demonstrating that CtBP1 can be sumoylated *in vitro* and *in vivo* at lysine 428 (Kagey et al., 2003; Lin et al., 2003). Mutation of Lys428 abrogates CtBP1 sumoylation and reduces repression activity. Examination of functional consequences of CtBP1 sumoylation revealed that this post-translational modification did not affect CtBP interaction with transcription factors, but rather blocked CtBP1 nuclear localization: the sumoylation-deficient mutant was largely localized in the cytoplasm. The $CtBP_L$ isoform in *Drosophila* also contains two putative SUMO motifs in the distal C terminus (Mani-Telang and Arnosti, 2007), and a systematic identification of sumoylated proteins in *Drosophila* confirmed that CtBP can indeed be sumoylated (Nie et al., 2009).

Phosphorylation may also play a role in regulating CtBP function. Using a yeast two hybrid system, Barnes and colleagues identified CtBP as an interacting protein of the p21-activated kinase 1 (Pak1). Functional studies showed that Pak1 phosphorylates CtBP1 at Ser158, triggers redistribution of CtBP from nucleus to cytoplasm, and suppresses its repression activity (Barnes

et al., 2003). Furthermore, CtBP1 has also been shown to be phosphorylated at Ser422 by the homeodomain interacting protein kinase 2 (HIPK2) (Zhang et al., 2005; Zhang et al., 2003). UV irradiation induced HIPK2-dependent phosphorylation of CtBP at Ser422, which in turn promoted proteasome-mediated CtBP degradation through the ubiquitin pathway, and triggered apoptosis. Interestingly, reduction in CtBP levels by genetic knockout or siRNA also promoted apoptosis in p53-deficient cells, indicating the UV-induced apoptosis in cells lacking p53 may function through degrading CtBP levels.

1.3 CtBP functions in development and disease

CtBP plays vital roles in animal development. Following the identification of CtBP in mammalian cells, the biological function of CtBP has been extensively studied in *Drosophila*. Using yeast two-hybrid screens, the Parkhurst and Levine groups independently identified *Drosophila* CtBP as an interacting partner of Hairy and short-range repressors, Knirps and Snail (Nibu et al., 1998b; Poortinga et al., 1998). Expression analysis showed that *Drosophila* CtBP is expressed throughout development and ubiquitously in early embryos. They both identified a *P*-element induced CtBP mutant allele, $CtBP^{03463}$, which is homozygous lethal. CtBP zygotic mutant animals die in the late larval stage, indicating that CtBP is required for early development. CtBP maternal mutant embryos exhibit severely disrupted segmentation and loss of ventral tissues, and die as embryos (Perrimon et al., 1996; Poortinga et al., 1998).

Drosophila embryonic body patterning is established during early embryogenesis by a cascade of gene regulation involving maternal, gap, pair-rule, and segment polarity genes (Nusslein-Volhard and Wieschaus, 1980). Each class of prior-acting "upstream" genes is expressed in relatively broader regions or stripes that are refined into more detailed patterns by

restricted expression of downstream gene products. For example, the primary pair-rule gene, *even-skipped* (*eve*), is expressed in a seven-stripe pattern. This particular expression pattern is controlled by gap genes including *knirps (kni) and Kruppel (Kr)*. Examination of early patterning gene expression in *CtBP* mutant embryos revealed that the pair-rule gene expression patterns, such as *eve* and *ftz*, were considerably altered, but the gap gene patterns, including *kni* and *Kr*, were essentially the same (Nibu et al., 1998a; Poortinga et al., 1998), indicating that CtBP may play direct roles in mediating repression activities of these repressors, rather than acting upstream. Indeed, the CtBP binding motif, PXDLS, is present in Knirps, Kruppel, and Snail repressors, and when mutated, these proteins show defective repression activity. Studies of *Drosophila* CtBP in the Levine group also suggested that CtBP may work as a short-range corepressor, together with short-range repressors such as Knirps, Kruppel, and Snail, in that the Knirps/Kruppel/CtBP pairs can only repress target genes over a short distance (<100 bp) (Mannervik et al., 1999). Interestingly, another corepressor, Groucho, interacts with long-range repressors Hairy and Dorsal, which can silence gene expression over a much longer distance (>1kb). This observation led to the proposal that Groucho and CtBP may mediate two distinct repression pathways in *Drosophila* embryos (Zhang and Levine, 1999). However, recent studies in our lab showed that the concept of long- and short-range repression in *Drosophila* is not that simple: Payankaulam et al. provided evidence showing that the long-range corepressor Groucho physically and genetically associates with the short-range repressor Knirps, and is a functional part of the Knirps repression complex (Payankaulam and Arnosti, 2009). Similarly, the short-range corepressor CtBP has also been shown to interact and play roles in Hairy-mediated gene silencing, which is long-range repression (Poortinga et al., 1998).

Genetic studies with knockout mice revealed that *CtBP* is also essential for vertebrate development. By generating different combinations of *CtBP1* and/or *CtBP2* null mice, Hildebrand and colleagues showed for the first time that mammalian *CtBP1* and *CtBP2* are likely to play overlapping and unique roles during mouse development (Hildebrand and Soriano, 2002). They disrupted *CtBP1* and *CtBP2* using homologous recombination and gene trapping technologies respectively, and showed in the $CtBP1^{+/-}$ $CtBP2^{+/-}$ heterozygous embryos that *CtBP2* is expressed uniformly at an early stage (embryonic day (E)7.5), but is more concentrated the in nervous system and ears and eyes at E9.5 to E12.5; while *CtBP1* is more widely expressed overall. Previous studies by the Higashi group also revealed that *CtBP2* is mainly expressed during embryogenesis, predominantly in the cephalic ganglia, spinal cord, and the limb buds, while *CtBP1* is detected from embryo to adult, expressed broadly among multiple tissues, but significantly in spinal cord (Furusawa et al., 1999). Consistent with their differential expression patterns, the $CtBP1^{-/-}$ and $CtBP2^{-/-}$ mice exhibit different phenotypes. Although a quarter of *CtBP1* null mice died by postnatal day 20 (P20), the majority of the mutants were viable and fertile, although they were about 30% smaller than their wild type and heterozygous littermates. In contrast, knocking out *CtBP2* is lethal: no homozygous offspring were recovered from the mating of *CtBP2* heterozygous mice; all *CtBP2* null mutant embryos die by E10.5. The mutant embryos showed multiple developmental defects, such as axial truncations, aberrant heart formation, altered neural structures, and defective extraembryonic vascularization. *CtBP1* and *CtBP2* interact genetically. Although the majority of $CtBP1^{+/-}$ $CtBP2^{+/-}$ mice are viable, the $CtBP1^{-/-}CtBP2^{+/-}$ mouse has a more severe phenotype than the $CtBP1^{-/-}$ knockout alone, in that mutants die as embryos. Similarly, disrupting the last copy of *CtBP2* in the compound

heterozygous background (*CtBP1*$^{+/-}$*CtBP2*$^{-/-}$) increases the phenotypic severity. A total knock out of *CtBP1* and *CtBP2* (*CtBP1*$^{-/-}$*CtBP2*$^{-/-}$) results in the most severe phenotypes. Despite these severe whole-organismal phenotypes, mouse embryonic fibroblasts completely lacking *CtBP1* and *CtBP2* can be recovered and cultured *in vitro*. A luciferase assay comparing repression activity of Gal4-Znf219, a transcription repressor whose repression is mediated by CtBP, in *CtBP1*$^{+/-}$ *CtBP2*$^{+/-}$ versus *CtBP1*$^{-/-}$ *CtBP2*$^{-/-}$ cells revealed that the repression potential of Gal4-Znf219 was impaired dramatically in *CtBP* null cells (Hildebrand and Soriano, 2002), indicating that functions of certain transcription factors are severely affected in the absence of CtBP, which may attribute to the *CtBP* null phenotypes.

CtBP may also play a role in the regulation of life span. The genetic studies carried out in *Drosophila* and mouse demonstrated that *CtBP* is essential for development. However, in *C. elegans*, *CtBP* is not required for development (Chen et al., 2009). A *C. elegans CtBP-1* mutant, *ok498*, in which the majority of the dehydrogenase domain is deleted, not only survives but exhibits an extended the life span (20% longer). Depletion of *CtBP-1* in the wild-type strain by RNAi resulted in a similar life span extension, while re-expression of *CtBP-1* in the mutant background rescued the life span phenotype. Consistent with this phenotype, the mutant worms showed increased resistance to oxidative and heat stress, a common feature of long-lived worms. A transcriptome analysis identified a putative triacylglycerol (TAG) lipase gene *lips-7* which may play a role in the life span phenotype associated with the loss of *CtBP-1*, in that depletion of *lips-7* suppressed the phenotype. However, the detailed mechanism is elusive.

It is surprising that the mutation has such distinct effects in development of *C. elegans* as compared with other organisms. Structurally, the *C. elegans* CtBP protein contains a unique N-

terminal Thanatos-associated protein (THAP) domain (Liew et al., 2007; Nicholas et al., 2008). The THAP domain shows sequence homology to the the *Drosophila P* element transposase DNA-binding domain, but its function is unknown. Human THAP1 and THAP7 have been suggested to play roles in transcriptional regulation. Their THAP domains have been shown to bind to specific sequences in DNA (THAP1) and to HDAC3 (THAP7) (Clouaire et al., 2005; Macfarlan et al., 2005). Interestingly, the THAP domain in *C. elegans* CtBP-1 is also able to bind to DNA, making it the first CtBP protein containing intrinsic DNA-binding activity (Liew et al., 2007). This unique THAP domain may therefore confer additional functions which might lead to the unique functions of CtBP in *C. elegans*, making it a distinct member in the CtBP family.

The potential roles of *CtBP* in oncogenesis stem from studies with the adenovirus *E1A* gene. *E1A* was originally considered as an oncogene in that it is capable of immortalizing primary rodent cells and transforming them in cooperation with adenovirus *E1B* or activated *ras* oncogene (Houweling et al., 1980; Ruley, 1983). E1A protein does not contain a DNA-binding domain or possess enzymatic activities, but instead functions by interacting with cellular proteins and modulating their activity. There are four conserved regions (CR1-4) in E1A that allow it to bind to a variety of factors including Rb, CBP/p300, and TBP (Frisch and Mymryk, 2002). The CR4 in the C-terminus contains a conserved PXDLS motif that permits interaction with CtBP (Boyd et al., 1993; Schaeper et al., 1995). Interestingly, E1A mutants lacking the CR4 or the PXDLS region have increased activity in *ras* cooperative transformation assays, and the transformed cells are less adherent, highly tumorigenic and metastatic (Schaeper et al., 1995; Subramanian et al., 1989), indicating that CtBP plays important roles in *E1A/ras* mediated transformation.

E1A may interfere with CtBP activity in a manner similar to that by which it targets the p300/CBP and p300/CBP-associated factor (PCAF) transcriptional coactivators. These proteins are directly bound by E1A and their histone acetyltransferase (HAT) activity is blocked, interfering with transcriptional activation (Chakravarti et al., 1999; Hamamori et al., 1999). Similarly, E1A-CtBP binding may sequester CtBP and de-repress CtBP target genes (Alpatov et al., 2004; Glasspool et al., 2005). E1A activates two telomerase genes, *hTERT* and *hTERC,* and this activation potential is dependent on the E1A CtBP interacting motif (Glasspool et al., 2005). Similarly, a nuclear speckle-associated protein Pnn/DRS also interacts with CtBP and attenuates CtBP-mediated repression of *E-cadherin* gene expression (Alpatov et al., 2004).

To obtain a global view of potential CtBP target genes, a transcriptome study compared gene expression profiles in *CtBP* null mouse embryo fibroblasts (MEFs) and *CtBP*-rescued MEFs. Targets of CtBP repression included epithelial genes such as *E-cadherin*, *plakoglobin*, *occluding* and *keratin-8*, and proapoptotic genes, such as *PERP*, *PTEN*, *p21*, *Bax*, and *Noxa* (Grooteclaes et al., 2003).. Consistent with the upregulated proapoptotic program, the *CtBP* null MEFs were hypersensitive to apoptosis. E1A activates epithelial gene expression and induces epithelial phenotypes (Frisch, 1994; Grooteclaes and Frisch, 2000), further supporting the model that E1A modulates oncogenesis by releasing CtBP-mediated target gene repression.

The identification of epithelial genes, particularly *E-cadherin*, as CtBP targets further emphasizes the importance of CtBP in tumorigenesis and tumor progression. E-cadherin is one of the most important adhesion molecules for cell-cell adhesion junctions and the formation and maintenance of epithelia. Loss of *E-cadherin* expression is a hallmark of epithelial-mesenchymal transitions (EMT), a process that is essential for embryonic development, but also implicated in tumor progression (Thiery, 2002; Thiery et al., 2009). The expression of *E-cadherin* is tightly

regulated during development by several developmental regulators, such as Snail (Cano et al., 2000), Twist (Yang et al., 2004), and ZEB1/2 (Comijn et al., 2001). Notably, Snail and ZEB1/2 have been shown to contain a CtBP-binding motif and require recruitment of CtBP for full function (Nibu et al., 1998a; Postigo and Dean, 1999). Furthermore, ZEB1/2 copurifies with CtBP (Shi et al., 2003), and must bind CtBP to repress *E-cadherin* expression (Grooteclaes and Frisch, 2000). High levels of *ZEB1* and *CtBP* expression have been strongly correlated with low levels of *E-cadherin* in colon carcinomas (Pena et al., 2006). ZEB-CtBP complex-mediated *E-cadherin* repression is further regulated by cellular redox status through CtBP's redox sensor function (Zhang et al., 2002; Zhang et al., 2006). Hypoxia, which increases cellular NADH levels, stimulates ZEB-CtBP interactions, recruitment of CtBP to the *E-cadherin* promoter, and repression of *E-cadherin* expression, which in turn reduces cell-cell adhesion, and increases cell mobility and invasiveness. However, addition of pyruvate, which reduces NADH levels, or knocking down CtBP, blocked hypoxia induced tumor cell migration (Zhang et al., 2006). Taken together, these studies strongly demonstrated the importance of CtBP in tumorigenesis through regulating *E-cadherin* expression.

In addition to *E-cadherin*, CtBP may also play roles in regulating other tumor suppressor genes. The systematic gene expression study performed by Grooteclaes et al. in *CtBP* null MEFs identified *PTEN* as a CtBP target (Grooteclaes et al., 2003). Interestingly, a reverse correlation between CtBP and PTEN protein levels was also observed by others. Overexpression of *CtBP* has been reported to be associated with decreased *PTEN* expression levels (Paliwal et al., 2007). It is not clear yet which repressor CtBP may work together with to repress *PTEN*, but a recent study showed that *PTEN* might be repressed during gamma-radiation induced apoptosis by Snail (Escriva et al., 2008), a repressor that requires CtBP, as suggested previously (Nibu et al., 1998a).

Although a direct interaction between CtBP and mammalian Snail is not evident (Bailey et al., 2007), studies have suggested that they may associate indirectly through other unknown factors (Mittal et al., 2008; Peinado et al., 2007; Tripathi et al., 2005). Recent studies also revealed that *p16*^{INK4A}, a tumor suppressor that is often inactivated in cancer and that plays roles in aging, is regulated by CtBP, as a CtBP knockdown increases p16^{INK4A} protein levels (Kim and Sharpless, 2006). CtBP occupies the *p16*^{INK4A} promoter, and hypoxia, which enhances CtBP activity, diminishes *p16*^{INK4A} expression (Zhang et al., 2002; Zhang et al., 2006). This regulation can be disrupted by oncoproteins; the E1A protein induces *p16*^{INK4A} expression in primary human fibroblasts and keratinocytes, and this induction by E1A is dependent on interaction with CtBP (Mroz et al., 2008) Repression of *p16*^{INK4A} by two Epstein-Barr virus latency-associated proteins, EBNA3A and EBNA3C, also requires CtBP (Skalska et al., 2010). Interestingly, CtBP may be directly targeted by a tumor suppressor. The ARF tumor suppressor encoded by the *INK4A/ARF/INK4B* locus, which also encodes p16^{INK4A}, interacts with CtBP and promotes proteasome-dependent CtBP degradation (Paliwal et al., 2006). The induction of apoptosis and prevention of cancer cell invasion by ARF is suggested to be mediated through the negative regulation of CtBP (Chen et al., 2008; Paliwal et al., 2007; Paliwal et al., 2006).

The relevance of CtBP in human disease further extends to regulation of the highly conserved Wnt signaling pathway that plays essential roles in development and is misregulated in human cancers (Polakis, 2000). In the canonical Wnt signaling pathway, in the absence of Wnt stimulation, the β-catenin transcriptional coactivator is targeted for destruction by the APC complex in a proteasome-mediated process. Upon Wnt stimulation, turnover is blocked, resulting

in accumulation and translocation of β-catenin into the nucleus, where it binds the TCF transcription factors to activate target gene expression (Cadigan, 2002). Interestingly, CtBP was found to be strongly associated with APC, and it is suggested that CtBP/APC binds to and diverts nuclear β-catenin away from TCF to reduce signaling (Hamada and Bienz, 2004). In addition, this APC-CtBP interaction has also been suggested to facilitate APC-mediated repression of TCF targets by recruiting CtBP corepressor complex to Wnt enhancers (Sierra et al., 2006). Consistent with repression function of CtBP on Wnt targets, Fang et al. have provided evidence that CtBP directly represses Wnt targets, such as *nkd*, in *Drosophila*. Interestingly, they also propose that CtBP may directly activate Wnt targets in a context-dependent manner, a novel activation role for CtBP in the Wnt signaling pathway (Fang et al., 2006). And the gene implicated in tumorigenesis that is regulated by CtBP is the deacetylase *SIRT1*, which plays important roles in aging-associated pathologies (Deltour et al., 2002; Herranz and Serrano, 2010; Van Rechem et al., 2010; Zhang et al., 2007). It has been shown that the tumor suppressor HIC1 physically associate with CtBP and represses SIRT1 expression in a CtBP-dependent manner (Deltour et al., 2002; Van Rechem et al., 2010; Zhang et al., 2007). *BRCA1,* a tumor suppressor gene often found to be mutated in breast cancer, is also found to be a target of CtBP (Di et al., 2010; Fackenthal and Olopade, 2007). Upon estrogen induced *BRCA1* activation, Di and colleagues observed that the CtBP/HDAC1 corepressor complex was dismissed and elongation factors were recruited to the *BRCA1* promoter. Depletion of CtBP from the BRCA1 promoter by CtBP siRNA or increased NAD^+/NADH level similarly decreased HDAC occupancy, increased histone acetylation and BRCA1 mRNA level, indicating that CtBP represses BRCA1 expression in the absence of estrogen (Di et al., 2010). Notably, the redox sensor activity of CtBP appears to be an important mechanism of modulating the expression of *BRCA1* and *SIRT1*. For example,

treatment of cells with 2DG, which inhibits glycolysis and thus decreases nuclear NADH levels, decreases association of CtBP with HIC1 and increases *SIRT1* expression (Zhang et al., 2007), as well as dissociates CtBP from the *BRCA1* promoter and induces *BRCA1* transcription (Di et al., 2010).

1.4 Molecular mechanisms of CtBP-mediated transcriptional repression

An emerging theme of corepressor-mediated transcriptional repression proposes that corepressors function as scaffold proteins bridging chromatin-modifying enzymes and sequence-specific transcription factors (Perissi et al., 2010) (Figure 2A). These corepressor complexes generally contain histone deacetylases, such as HDAC1/2, which functionally execute repression activity by deacetylating histone tails and forming repressive chromatin structures when recruited to target promoters. Several lines of evidence indicate that histone deacetylases play important roles in CtBP-mediated repression. CtBP can interact directly with histone deacetylases HDAC1/2/3 and trichostatin A (TSA), a histone deacetylase inhibitor, significantly reduces CtBP repression (Criqui-Filipe et al., 1999; Subramanian and Chinnadurai, 2003; Sundqvist et al., 1998). Direct purification of CtBP from mammalian cells identified HDAC1 and HDAC2 as core components; the purified complex exhibited significant HADC activity *in vitro* (Shi et al., 2003). Other histone modifiers were also identified in the complex including histone methyltransferases (HMT) G9a and Eu-HMTase1 (EuHMT), and the histone demethylase LSD1 (Shi et al., 2004). The CtBP complex contains HMT activity, and depletion of G9a or EuHMT relieved repression of the *E-cadherin* promoter by CtBP. Shi and colleagues proposed a model in which the CtBP complex may silence gene expression by switching the histone H3K9-acetyl active mark to the H3K9-methyl repressive mark by the sequential action of

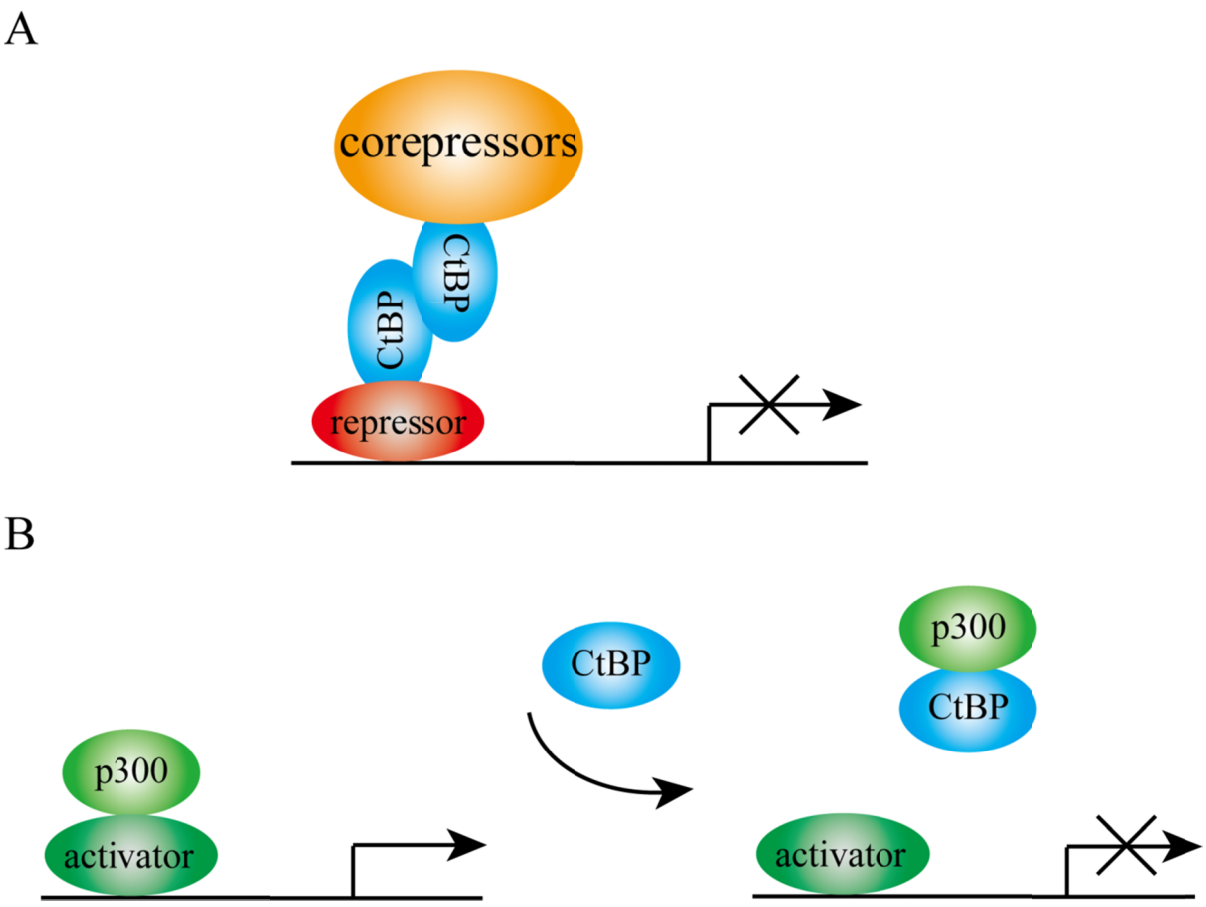

Figure 2. Mechanisms of CtBP-mediated transcriptional repression. (A) CtBP represses gene expression by recruiting chromatin-modification enzymes to target gene promoters. The dimeric CtBP recruits chromatin-modifying enzymes, depicted as corepressors in orange, including HDACs, HMTs, and LSD1 (Shi et al., 2003) through one substrate binding domain, and targets the corepressor complex to a promoter by associating with a sequence-specific repressor via the second domain. These enzymes coordinately modify histone marks to form a repressive chromatin structure. (B) Alternatively, CtBP may inhibit the HAT activity of p300 activator by interacting directly with its bromodomain and dissociating it from activator.

HDACs and HMTs (Shi et al., 2003). Although the histone demethylase LSD1 is also a constituent of CtBP complexes, its role in CtBP-mediated repression has not been fully evaluated yet.

If CtBP were to recruit histone modification enzymes to DNA-bound transcription factors in order to carry out gene repression, then CtBP should bind simultaneously to sequence-specific transcription repressors and histone modifiers. CtBP interacts with transcription factors mainly through the substrate binding domain that recognizes the conserved PXDLS CtBP-binding motif, however, the same domain is also responsible for recruiting enzymatic components of the complex (Kuppuswamy et al., 2008). This model suggests that dimerization would be important for CtBP function, so that one protein binds a transcription factor, while its partner interacts with histone modification enzymes. Indeed, although a monomeric CtBP mutant was able to interact effectively with transcription factors as well as histone modifiers, it failed to repress target gene expression (Kuppuswamy et al., 2008), emphasizing the significance of CtBP dimerization. In support of this model, crystal structure studies clearly showed that CtBP forms a dimer (Figure 1C) (Kumar et al., 2002; Nardini et al., 2003). The dimerization is mainly mediated by the dehydrogenase domain, leaving the two substrate binding domains at the two extremes, which may facilitate the binding of transcription factors and enzymatic components simultaneously.

An additional route by which CtBP may repress target genes is by antagonizing transcriptional coactivators. The p300/CBP and PCAF transcriptional coactivators are histone acetyltransferases that acetylate lysine residues to relax the chromatin structure and facilitate binding of stimulatory factors (Roth et al., 2001). Interestingly, three groups reported independently in 2005 that CtBP specifically interacts with p300/CBP and PCAF coactivators to inhibit their HAT activity (Kim et al., 2005; Meloni et al., 2005; Senyuk et al., 2005). They

showed that the p300/CBP and PCAF coactivators contain potential CtBP PMDLS binding motifs in their bromodomains that mediate the CtBP-CBP interaction (Kim et al., 2005), although one group reported the interaction was independent of the CBP bromodomain (Senyuk et al., 2005). The bromodomain associates with acetylated histone residues directly, and it was suggested that binding of CtBP to the bromodomain blocked interactions between the bromodomain and acetylated histones, inhibiting p300/CBP-mediated histone acetylation and gene activation (Kim et al., 2005; Meloni et al., 2005; Senyuk et al., 2005). Notably, Kim et al. observed that the binding of CtBP to p300/CBP was negatively regulated by NADH: while the interactions between the bromodomain and histones were effectively inhibited by CtBP, the inhibitory effect was ablated by addition of NADH. CtBP mutants that were unable to bind NADH or to dimerize showed the strongest inhibitory effect on p300/CBP-mediated histone acetylation and transcription activation. Apparently, the monomeric form of CtBP interacts with p300/CBP more effectively, and thus NADH, which stimulates CtBP dimerization, may disassociate CtBP from p300/CBP and relieve CtBP-mediated inhibition of p300/CBP activity (Balasubramanian et al., 2003; Nardini et al., 2009; Thio et al., 2004),.

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CHAPTER 2

CONSERVED CATALYTIC AND C-TERMINAL REGULATORY DOMAINS OF THE CTBP COREPRESSOR FINE TUNE TRANSCRIPTIONAL RESPONSE IN DEVELOPMENT

Abstract

Transcriptional corepressors play complex roles in developmental gene regulation. These proteins control transcription by recruiting diverse chromatin modifying enzymes, but it is not known whether corepressor activities are finely regulated in different developmental settings, or whether basic activities are identical in most contexts. The evolutionarily conserved C-terminal binding protein (CtBP) is recruited by a variety of transcription factors that play crucial roles in development and disease. CtBP contains a central NAD(H)-binding core domain homologous to D2 hydroxy acid dehydrogenase enzymes, as well as an unstructured C-terminal domain. NAD(H) binding is important for CtBP function, but the significance of its intrinsic dehydrogenase activity, as well as the unstructured C-terminus, is poorly understood. To clarify the biological relevance of these features, we established genetic rescue assays to determine how different forms of CtBP function in the context of *Drosophila* development. Mutant phenotypes and specific gene regulatory effects indicate that both the catalytic site of CtBP, as well as the C-terminal extension play important, if nonessential roles in development. Our results indicate that the structural and enzymatic features of CtBP, previously thought to be dispensable for overall transcriptional control, are critical for modulating this protein's activity in diverse developmental settings.

Introduction

Developmental gene regulation features an elaborate interplay of transcription activation and repression. A variety of transcriptional cofactors are required in eukaryotes to potentiate the activity of sequence-specific transcription factors that effect these regulatory programs. Cofactors often are crucial for alteration of chromatin structures at target genes; corepressor complexes involved in gene inactivation have various enzymatic activities, including nucleosome remodeling, histone deacetylation, methylation, and demethylation. Although the importance of corepressors in transcriptional repression has been appreciated at the cellular level, the biological functions of corepressors in the development of multicellular organisms are less well understood.

The C-terminal binding protein (CtBP) is an evolutionarily conserved transcriptional corepressor that plays crucial roles in development and disease (Chinnadurai, 2002, 2009). Initially identified as a phosphoprotein associated with the C-terminus of the adenovirus E1A protein, the CtBP dimer has been shown to interact with a variety of cellular transcription factors containing a PXDLS motif to regulate target genes implicated in multiple cellular processes (Turner and Crossley, 2001). *Drosophila* CtBP is utilized by transcriptional repressors such as the Knirps, Krüppel, Giant, and Snail proteins that are active in patterning the blastoderm embryo (Nibu et al., 1998a; Poortinga et al., 1998). Structurally, CtBP shares extensive homology to D-2 hydroxy acid dehydrogenases, including the conserved NAD(H)-binding domain and putative catalytic site, and the protein has weak *in vitro* dehydrogenase activity (Kumar et al., 2002). Although CtBP active site residues are highly conserved, it is not clear whether the dehydrogenase activity participates in CtBP-mediated transcriptional repression, because most cell-based assays have shown that this enzymatic activity is dispensable for

repression (Grooteclaes et al., 2003; Kumar et al., 2002; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004). The NAD(H)-binding activity, however, appears to be critical for CtBP repression activity (Grooteclaes et al., 2003; Kumar et al., 2002; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004). Structural studies revealed that NAD(H) binding induces a conformational change in CtBP which may explain the importance of this cofactor for the interaction of CtBP with cofactors and transcription factors (Kumar et al., 2002; Zhang et al., 2002). A NAD(H) binding mutant is still capable of forming dimers *in vitro* but evidence suggests that NAD(H) binding can further stimulate CtBP dimerization (Balasubramanian et al., 2003; Mani-Telang et al., 2007).

Like other corepressors that serve as scaffolds to recruit chromatin-remodeling enzymes, CtBP diversity is increased by gene duplication, alternative splicing and post-translational modification (Payankaulam et al., 2010). In invertebrates and vertebrates, multiple CtBP isoforms are expressed in spatially and temporally differentiated patterns, suggesting specialization of function. In *Drosophila*, the single *CtBP* gene encodes two major isoforms generated through alternative RNA splicing (Nibu et al., 1998b; Poortinga et al., 1998; Sutrias-Grau and Arnosti, 2004). These isoforms, termed $CtBP_L$ and $CtBP_S$, are highly conserved in insects (Mani-Telang and Arnosti, 2007). The proteins are identical at the N-terminus but differ in the C-terminal region: $CtBP_L$ has an extended C-terminus, which is lacking in $CtBP_S$. Tethered directly to DNA, these two isoforms exhibit similar repression activity, but have distinct developmental expression profiles, suggesting they may have overlapping or unique functions (Mani-Telang and Arnosti, 2007; Sutrias-Grau and Arnosti, 2004). The vertebrate *CtBP1* and *CtBP2* genes also produce various isoforms via either RNA splicing or alternative promoter utilization (Verger et al., 2006). CtBP1-L and CtBP1-S are encoded by the *CtBP1* gene

through alternative splicing, while CtBP2 and RIBEYE are produced from the *CtBP2* gene by alternative promoter usage. CtBP1-L and CtBP2 exhibit corepressor activities, while CtBP1-S may be involved in Golgi fission, and RIBEYE in the function of central nervous system synapses (Schmitz et al., 2000; Weigert et al., 1999).

The unstructured C-terminal domain is present in all vertebrate CtBP proteins and the $CtBP_L$ isoform in *Drosophila* (Nardini et al., 2006). This region contains sites for posttranslational modifications including phosphorylation and sumoylation, which may play regulatory roles: C-terminal phosphorylation of human CtBP1-L triggers CtBP1-L ubiquitylation and degradation, while sumoylation of this tail regulates CtBP1-L subcellular distribution by stimulating nuclear retention (Lin et al., 2003; Zhang et al., 2005; Zhang et al., 2003). A further level of regulation is mediated by acetylation of a lysine residue near the N-terminus of mammalian CtBP2, which can control nuclear localization (Zhao et al., 2006).

Similar to other corepressors, CtBP also forms complexes with multiple chromatin modifying enzymes. Proteomic studies have identified histone deacetylase, histone methyl transferase, and histone demethylase proteins in CtBP complexes, suggesting that CtBP works as a platform to recruit chromatin modifiers to the template to alter chromatin structure and repress target gene expression (Shi et al., 2003). CtBP may also play a more complex role than merely serving as a scaffold to bridge transcription factors and histone modifiers. The intrinsic dehydrogenase activity of CtBP may participate in transcriptional regulation, although the significance of this function has remained elusive. In addition, CtBP may also work as a sensor to monitor cellular redox status and regulate transcription accordingly (Fjeld et al., 2003; Zhang et al., 2002). Both NADH and NAD^+ stimulate CtBP-repressor interaction, but the binding affinity for the reduced NADH cofactor has been measured to be >100 fold higher than affinity for NAD^+, which may

permit CtBP to respond to changes in balance of these two forms of dinucleotide, providing a coupling between metabolism and transcription (Fjeld et al., 2003; Kumar et al., 2002; Zhang et al., 2002). Kumar et al. and Balasubramanian et al., however, did not observe the different efficiency of NADH and NAD^+ in stimulating CtBP-E1A interaction in in vitro binding assays, thus there is some uncertainty regarding relative binding affinity to the reduced and oxidized dinucleotides (Balasubramanian et al., 2003; Kumar et al., 2002).

Cell-based studies have shed light on basic repression activities of CtBP, but the functions of CtBP in the context of development are less well understood. *CtBP* is an essential gene for development in *Drosophila*, as is the *CtBP2* gene in mouse (Hildebrand and Soriano, 2002; Nibu et al., 1998a; Poortinga et al., 1998). Murine *CtBP1* mutants are viable but show growth defects, and in *C. elegans*, *CtBP* mutants do not die but in fact exhibit an extended life span (Chen et al., 2009; Hildebrand and Soriano, 2002). These studies provide only a very general picture of CtBP functions on a global scale, but do not address how specific features of the protein may contribute qualitatively or quantitatively to gene regulation in specific contexts. To better understand the biological relevance of different features of CtBP in a developmental context, we generated genomic rescue constructs that gave us the ability to assay different CtBP forms expressed under control of native *cis* regulatory elements in a *CtBP*-null background, providing the first such detailed dissection of CtBP function in a whole organism. The mutant phenotypes indicate that the putative dehydrogenase function, as well as the C-terminal regulatory domain, are in fact essential for normal developmental activity of this corepressor.

Materials and methods

***CtBP* rescue constructs.** The 16 kb annotated *Drosophila CtBP* gene region was divided into three pieces, named *CtBP*-1st, *CtBP*-2nd, and *CtBP*-3rd, and amplified from *yw* adult genomic DNA, using Expand Long Range dNTPack kit (Roche). BamHI/AvrII, AvrII/BglII, and BglII/KpnI sites were designed into 5' and 3' ends of *CtBP*-1st, *CtBP*-2nd, and *CtBP*-3rd, respectively. A pair of oligonucleotides containing BamHI-AvrII-BglII-KpnI sites were annealed and inserted into BamHI/KpnI sites of pBluescript vector for assembly (pBS-adaptor). The *CtBP*-2nd fragment was first ligated into T-easy vector (Promega) and used as template to generate *CtBP-CAT* (H315Q) and *CtBP-NAD* (D204N) mutations (Figure 3D) via QuikChange Site-Directed Mutagenesis (Stratagene). Two tandem Flag tags (2xFlag) were inserted, in frame, to C-termini of the last protein coding regions of $CtBP_S$ and $CtBP_L$ using QuikChange strategy. The modified fragments were assembled in pBS-adaptor in the order of *CtBP*-2nd, *CtBP*-1st, and *CtBP*-3rd, and the whole gene region was then subcloned into pattB vector (a gift from K. Basler, University of Zurich, Switzerland) using BamHI/KpnI sites (BglII/KpnI for pattB vector). To generate the $CtBP_S$ construct, the splicing donor site at the end of exon 5 was mutated from G<u>T</u> to G<u>A</u>, and exons encoding the C-terminal extension of $CtBP_L$ were deleted (Figure 3C). The $CtBP_L$ construct was generated by fusing the C-terminal extension region to the C-terminal of $CtBP_S$ directly (Figure 3C).

Fly stocks. *CtBP* mutant lines *CtBP*^{03463} (P11590) and *CtBP*$^{87De-10}$ (1663), deficiencies *Df(3R)Exel8157* (7973) and *Df(3R)BSC615* (25690) were obtained from Bloomington stock

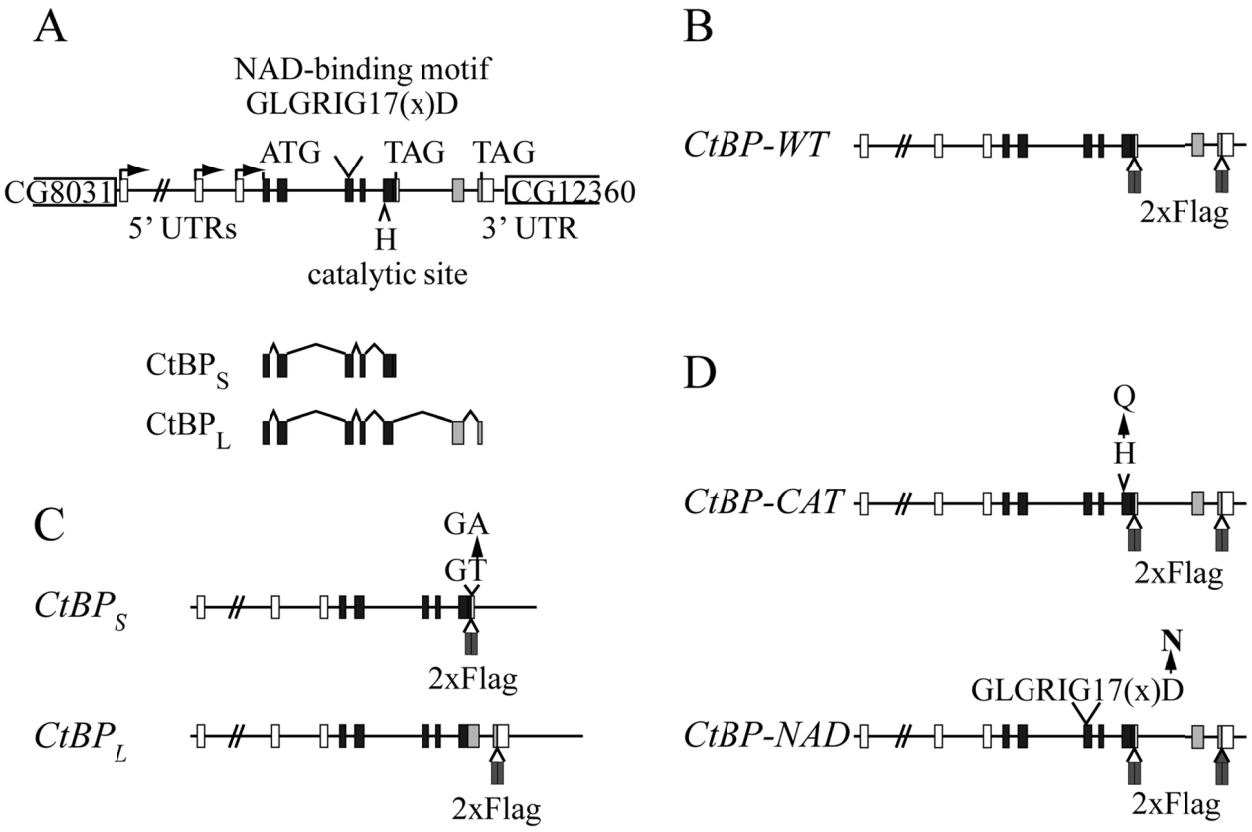

Figure 3. Structures of *CtBP* genomic constructs.

Figure 3 (cont'd)

Figure 3. Structures of *CtBP* genomic constructs. (A) Schematic representation (not to scale) of the exon structure of the genomic locus and splicing patterns of *Drosophila CtBP*. Protein coding exons present in both $CtBP_S$ and $CtBP_L$ are indicated in black, whereas the $CtBP_L$ C-terminal extension is highlighted in gray. Untranslated sequences of exons are in white. Amino acids of the conserved NAD-binding motif and catalytic site are indicated. (B to D) Schematic structure of genomic *CtBP-WT*, $CtBP_S$, $CtBP_L$, *CtBP-CAT* and *CtBP-NAD* rescue constructs. Tandem Flag tags (2xFlag) were fused to the C-termini of $CtBP_S$ and $CtBP_L$. (C) $CtBP_S$ is generated by mutating a splice donor site (silent mutation), and deleting exons encoding the C-terminus of $CtBP_L$. The same region is fused to the 3' end of $CtBP_S$ to generate $CtBP_L$. (D) To generate forms of the protein lacking catalytic function or NAD(H) binding activity, the conserved catalytic histidine codon is mutated to glutamine (H to Q) in the *CtBP-CAT* construct, and aspartic acid to asparagine (D to N) in the *CtBP-NAD* construct (Balasubramanian et al., 2003; Kumar et al., 2002; Kuppuswamy et al., 2008).

center and used for rescue assays. *CtBP* genomic constructs were injected into line attp40 using phiC31 integrase system at Genetic Services, Inc.. The cytogenetic location of *attB* site in line attp40 is 25C7.

Transient transfection and Western blotting. *Drosophila* Schneider 2 (S2) cells were grown at 25°C in Schneider *Drosophila* medium (GIBCO) containing 10% heat-inactivated FBS, 50 units penicillin G and 50 ug/ml streptomycin sulfate. For each transfection, ~1x10^6 cells were seeded per well in a six-well plate and transfected with 200 ng of each DNA using Effectene transfection kit (Qiagen). Cells were grown for three additional days and lysed in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), supplemented with Protease Inhibitor Cocktail (Roche). Protein concentration was measured by Bradford assay. Equivalent amount of total protein was subjected to SDS-PAGE (NuPAGE Novex Bis-Tris 4-12% gel) and analyzed by immunoblotting. Images and quantifications were acquired on a LAS-3000 imaging system (Fuji). Antibodies used in this study were mouse anti-Flag M2 antibody (Sigma, 1:10,000), rabbit anti-CtBP serum (1:10,000) (Mani-Telang and Arnosti, 2007), mouse anti-β-tubulin (1:5,000, Iowa Hybridoma Bank), goat-anti-mouse and goat-anti-rabbit HRP-conjugated secondary antibodies (Pierce, 1:10,000).

Immunofluorescent staining and confocal laser scanning microscopy. Immunofluorescent staining of *Drosophila* embryos was performed as previously described (Ay et al., 2008). Briefly, fixed embryos were washed six times in 100% ethanol, once in xylene for 30 min, six times in 100% ethanol, four times in 50% methanol/ 50% PBT (1x PBS + 0.1% Tween 80), four times in 100% PBT, and then blocked once in Blocking reagent (1% casein in maleic acid and PBT, 1:1, v/v) for 1 hour at RT with rocking. The embryos were then incubated with primary antibody and fluor-conjugated secondary antibody in blocking reagent overnight at 4°C, and mounted on

slides. Samples were examined using an Olympus FluoView 1000 laser scanning confocal microscope-IX81 at the Center for Advanced Microscopy at Michigan State University (MSU). Antibodies used were mouse anti-Flag M2 antibody (Sigma, 1:500), rabbit anti-CtBP serum (1:200), Alexa Fluor 555 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (1:500, invitrogen).

Scanning electron microscopy (SEM). Wings were removed from adult flies directly, mounted onto a metal stub, coated with gold and imaged with a JEOL 6400V Scanning Electron Microscope (Japan Electron Optics Laboratories) at the Center for Advanced Microscopy at MSU.

Genetic rescue assay. To move a *white* mutant allele into the $CtBP^{03463}$ stock, male flies (w^{+}/Y; $CtBP^{03463}/TM3$, $Sb^{1}Ser^{1}$) were crossed with *Ser* virgin females (w^{-}/w^{-}; +/*TM3, Ser*). Offspring carrying *white* and the $CtBP^{03463}$ mutant alleles (w^{-}/Y; $CtBP^{03463}/TM3$, *Ser* and w^{-}/w^{+}; $CtBP^{03463}/TM3$, *Ser*) were crossed *inter se* and progeny with white eyes and the *Ser* balancer (w^{-}/w^{-}; $CtBP^{03463}/TM3$, *Ser* and w^{-}/Y; $CtBP^{03463}/TM3$, *Ser*) were collected and maintained as new $CtBP^{03463}$ stock. The same crosses were performed to remove w^{+} gene in $CtBP^{87De\text{-}10}$ stock. To conduct the *CtBP* rescue assay, transgenic flies carrying individual transgene (tg) were first crossed with *TM3, Sb* flies. Male offspring (*tg/+; +/TM3, Sb*) were crossed with $CtBP^{03463}$ virgin females (w^{-}/w^{-}; $CtBP^{03463}/TM3$, *Ser*). Offspring containing the *CtBP* transgene and the $CtBP^{03463}$ mutant allele (*tg/+;* $CtBP^{03463}/TM3$, *Sb*) were crossed *inter se* to assess their abilities to rescue the *CtBP* null phenotype. The $CtBP^{87De\text{-}10}$ mutant was

similarly tested but we could not rescue this stock with any rescue construct. Suspecting that this stock may harbor an additional recessive lethal mutation, we performed additional complementation analysis, placing $CtBP^{87De-10}$ over $CtBP^{03463}$ or over two *CtBP* deficiencies, *Df(3R)BSC615* and *Df(3R)Exel8157*, and all of them could be rescued, suggesting that the $CtBP^{87De-10}$ chromosome may contain additional recessive lethal mutations not related to *CtBP*.

In situ hybridization. 2-4 hour embryos were collected, fixed and hybridized with digoxigenin-UTP labeled RNA antisense probe to *eve* (Small et al., 1992).

Real-Time PCR. Total RNA from rescued adult flies was extracted using RNeasy Mini Kit (Qiagen) following manufacturer's instructions, including on-column DNase digestion step. Three ug of total RNA was *in vitro* transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with RNase inhibitor in 40 ul volume. cDNA was then diluted into 25ng/4ul and 25ng of total cDNA was used in each 16 ul real-time PCR reaction. The final concentration of each primer was fixed to 250nM. SYBR Green PCR Master mix kit (Applied Biosystems) was used in this study, and real-time PCR was performed on Applied Biosystems 7500 real-time PCR system. PCR conditions were: 50°C 2 min, 1 cycle; 95°C 10 min, 1 cycle; 95°C 15 s, 60°C 1 min, 40 cycles. A dissociation step was added to determine primer specificity. Amplification efficiency (E) of each primer set was calculated by standard curve method, using 5 series of 5-fold dilutions (25ng/4ul, 5ng/4ul, 1ng/4ul, 0.2ng/4ul, and 0.04ng/4ul) (Pfaffl, 2001). Putative CtBP direct targets were selected based on physical binding of CtBP to gene promoter (*Drosophila* CtBP ChIP-chip data, http://intermine.modencode.org/release-18/objectDetails.do?id=211000586). Gene transcripts were measured in three biological replicates, two technical replicates for each sample, and

relative gene expression was determined using the Relative Expression Software Tool (Pfaffl et al., 2002), with β-*tubulin56D* (Fang et al., 2006) as reference gene.

Results

Expression of *Drosophila CtBP* from endogenous genomic regulatory sequences. The single *Drosophila CtBP* gene produces multiple isoforms through RNA splicing (Poortinga et al., 1998; Sutrias-Grau and Arnosti, 2004). Two major isoforms are expressed throughout development, with $CtBP_S$ expressed at higher levels than $CtBP_L$. To faithfully reproduce endogenous *CtBP* expression levels and patterns, we generated a 16 kb rescue construct containing 8 kb 5' and 1 kb 3' of the central coding exons, including a large 3 kb intron that is spliced out to form the $CtBP_L$ isoform. A C-terminal Flag epitope tag was used to track the expression of *CtBP* transgene products. Five transgene constructs, all inserted at the same locus to eliminate position effects, were assayed to determine the activity of CtBP functional elements (Figure 3). A wild-type construct, *CtBP-WT*, preserves the exon and intron structure, as do two mutant forms, *CtBP-CAT* and *CtBP-NAD*, with single point mutations eliminating catalytic activity or NAD(H) binding (Balasubramanian et al., 2003; Kumar et al., 2002; Kuppuswamy et al., 2008). To eliminate catalytic activity, the critical catalytic active site histidine was mutated to glutamine in the *CtBP-CAT* mutant, while NAD(H) binding was disrupted by an aspartic acid to asparagine mutation in the *CtBP-NAD* mutant (Figure 3). These mutations have been described in previous forms of CtBP (Kuppuswamy et al., 2008; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004) A previously described *CtBP-NAD* mutation affecting conserved glycines present in the NAD(H) binding Rossmann fold (GXGXXG) was not used in this study because of its strong destabilizing affect (Mani-Telang et al., 2007). A fourth gene, $CtBP_S$, produces only

the abundant short isoform, while *CtBP$_L$* joins the alternatively spliced 3' exon directly to the main coding region to produce this isoform at levels similar to that of the more abundant endogenous CtBP$_S$ isoform.

The expression of these genes was first assayed by transient transfection in *Drosophila* S2 cells followed by Western blotting with anti-Flag M2 antibody to detect the transgenic products. Both CtBP$_L$ and CtBP$_S$ isoforms were detected in *CtBP-WT*, *CtBP-CAT*, and *CtBP-NAD* transfections, with higher levels of the short isoform, as expected (Figure 4B, lanes 2, 5, and 6). Similarly, the *CtBP$_L$* construct produced only the long isoform, while the *CtBP$_S$* expressed solely the short variant (Figure 4B, lanes 3 and 4). Consistent with these results, transgenic flies carrying *CtBP-WT*, *CtBP-CAT* and *CtBP-NAD* transgenes expressed both CtBP$_L$ and CtBP$_S$ isoforms, whereas only CtBP$_L$ or CtBP$_S$ was detected in *CtBP$_L$* or *CtBP$_S$* transgenic flies (Figure 4C). To compare expression of the exogenous CtBP proteins to endogenous CtBP isoforms, extracts from lines expressing the proteins were analyzed with anti-CtBP antibody that recognizes both endogenous and recombinant proteins, allowing direct comparison of the levels of these proteins (Figure 4D). The exogenous CtBP isoforms were quantified and normalized to endogenous CtBP proteins in each sample. As shown in Figure 4E, the *CtBP-WT*, *CtBP$_L$*, *CtBP$_S$*, and *CtBP-CAT* transgenic lines produced amounts of proteins similar to the endogenous levels, while *CtBP-NAD* expression level was roughly one-half of this level, consistent with a lower stability of this mutant form (Mani-Telang et al., 2007).

CtBP and CtBP catalytic mutant, but not the CtBP-NAD binding mutant, localize to the nucleus. Endogenous CtBP is found primarily in the nucleus, consistent with its role as a

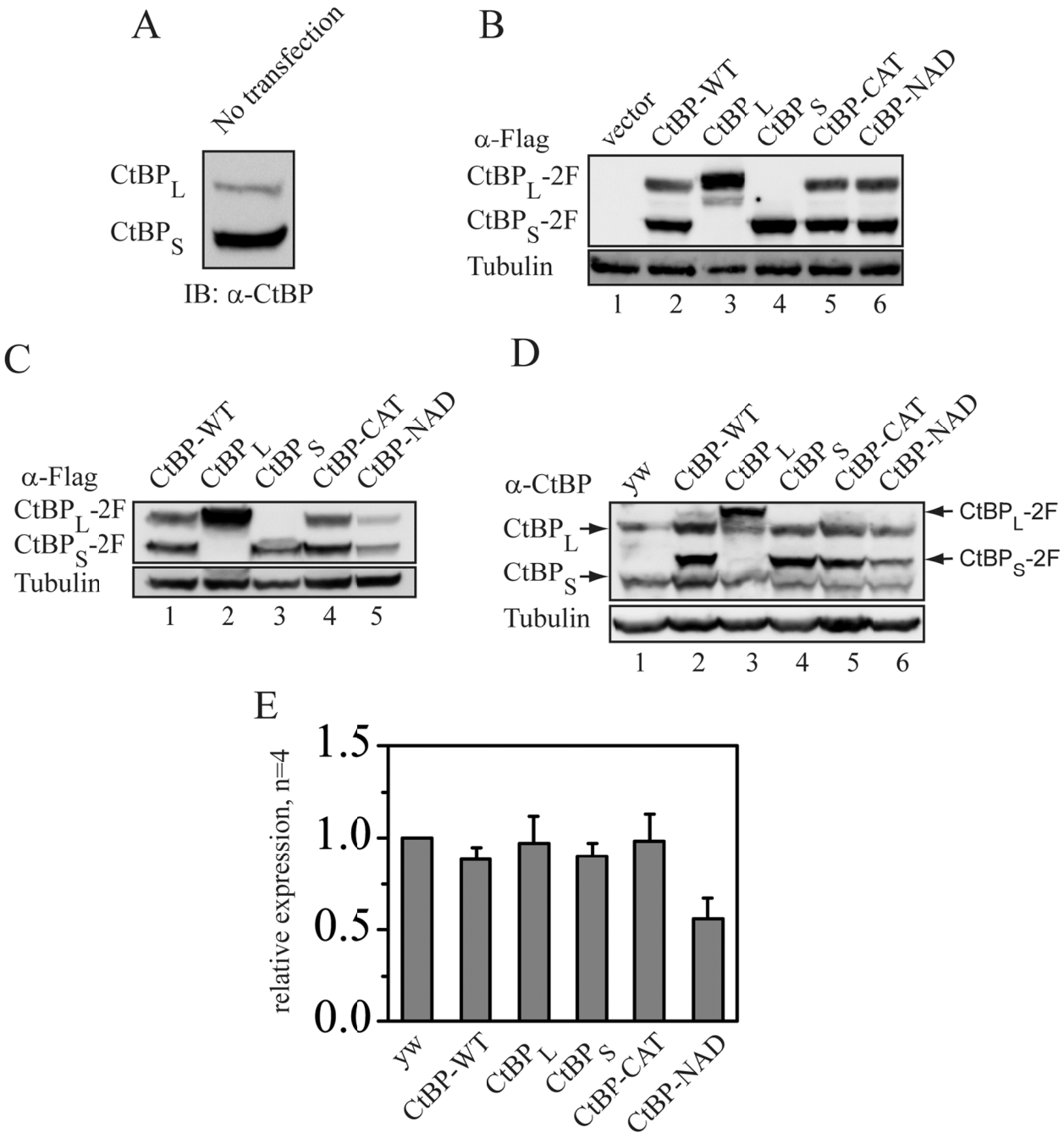

Figure 4. *CtBP* genomic constructs drive protein expression at physiological levels.

Figure 4 (cont'd)

Figure 4. *CtBP* genomic constructs drive protein expression at physiological levels. (A) Endogenous $CtBP_S$ and $CtBP_L$ expression in S2 cells. (B) Expression of *CtBP* rescue constructs in S2 cells. Cells were transfected with indicated rescue constructs for 3 days and lysates were analyzed by Western blotting using anti-Flag antibody. Both $CtBP_L$ and $CtBP_S$ isoforms were detected in *CtBP-WT*, *CtBP-CAT* and *CtBP-NAD* transfections, while only $CtBP_L$ or $CtBP_S$ was expressed by $CtBP_L$ or $CtBP_S$ constructs, as expected. (C) Expression of *CtBP* rescue constructs in transgenic flies. Adult fly extracts were subjected to Western blot analysis with anti-Flag antibody. CtBP-NAD binding mutants were expressed at reproducibly lower levels than those of other constructs (S2 cell expression of this protein was also usually lower, although not in the experiment shown in 2B). (D, E) Exogenous CtBP levels compared to endogenous levels in transgenic flies. Adult fly extracts were analyzed with anti-CtBP antibody to directly compare endogenous and exogenous proteins (Flag-tagged proteins migrate slower than endogenous CtBP proteins). (D) The exogenous ($CtBP_L$-2F + $CtBP_S$-2F) and endogenous ($CtBP_L$ + $CtBP_S$) CtBP isoforms were quantified separately in each lane and relative protein expression levels were calculated. (E) Expression of exogenous CtBP isoforms relative to endogenous CtBP proteins in transgenic flies. Quantifications were performed on four reproducible Western blots as shown in (D).

transcriptional corepressor. Accordingly, a nuclear localization signal (NLS) was identified in the N-terminus of *Drosophila* CtBP (Verger et al., 2006). To examine whether NAD(H) binding and the dehydrogenase activity affect protein localization, transgenic *Drosophila* embryos were stained with anti-CtBP or anti-Flag antibodies to visualize CtBP proteins. $CtBP_L$, $CtBP_S$, and CtBP-CAT (Fig 3B, C, and D) were all localized to the nucleus, as was endogenous CtBP (Figure 5A). Protein was detected in both the nucleus and cytoplasm in *CtBP-NAD* transgenic *Drosophila* embryos that also have endogenous CtBP (Figure 5E), and largely in the cytoplasm in embryos expressing only the NAD(H)-binding mutant form of CtBP (Figure 5F). We suspect the partial nuclear localization observed in the presence of endogenous CtBP is directed by heterodimerization between wild-type CtBP and CtBP-NAD mutant proteins; apparently NAD(H) binding is essential for CtBP nuclear localization.

***$CtBP_L$*, *$CtBP_S$*, and *CtBP-CAT*, but not *CtBP-NAD*, rescue a *CtBP* mutant.** We next carried out genetic rescue assays to test the ability of CtBP isoforms and mutants to rescue a *CtBP* null mutant, $CtBP^{03463}$, to assess their functions in a developmental context (Nibu et al., 1998a; Poortinga et al., 1998). $CtBP^{03463}$ is a P-element induced homozygous lethal mutant and the homozygotes die as pharate adults in the pupal case (Nibu et al., 1998b; Poortinga et al., 1998). Different forms of *CtBP* transgenes were introduced into *CtBP* homozygous mutants to first test whether they could functionally substitute for zygotic CtBP (Figure 6A). In the presence of rescue constructs, a considerable number of adult flies survived despite being homozygous for the *CtBP* mutation (Figure 6B). One or two copies of the $CtBP_L$, or $CtBP_S$ transgene were sufficient to rescue *CtBP* lethality. A smaller percentage of flies were rescued by the *CtBP-CAT*

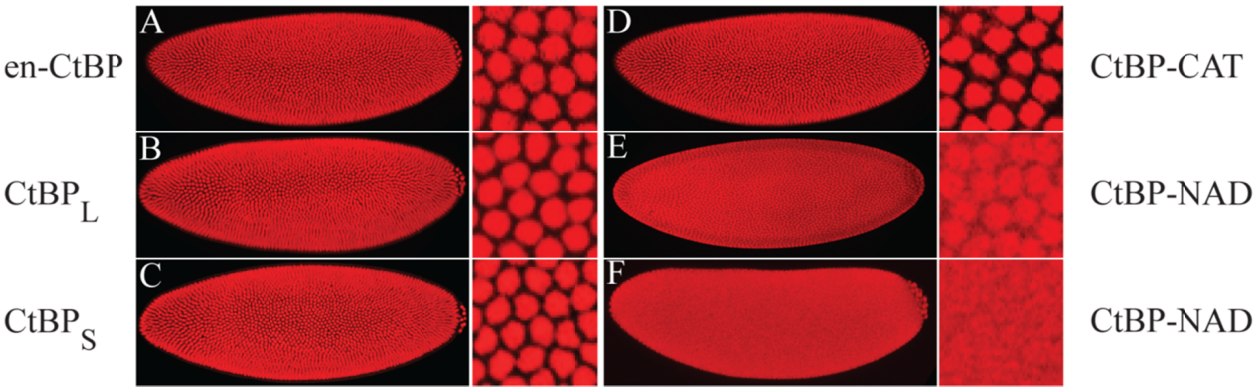

Figure 5. Localization of CtBP proteins. CtBP isoforms and catalytic mutants localize to the nucleus in embryos, while the NAD-binding mutant is largely cytoplasmic. (A) Localization of endogenous CtBP protein. (B-D) CtBP$_L$, CtBP$_S$, and CtBP-CAT proteins were found in the nucleus of transgenic embryos, both in the presence of endogenous CtBP shown here, and in rescued embryos lacking endogenous CtBP (data not shown). CtBP-NAD mutant proteins were detected in both the nucleus and cytoplasm in transgenic embryos also expressing endogenous CtBP (E), and mostly in the cytoplasm in *CtBP-NAD* mutant embryos (F). Transgenic embryos from each line (B-F) were stained with anti-Flag antibody and protein localization was visualized by confocal laser scanning microscopy. Wild-type non-transgenic embryos (A) were stained with anti-CtBP antibody.

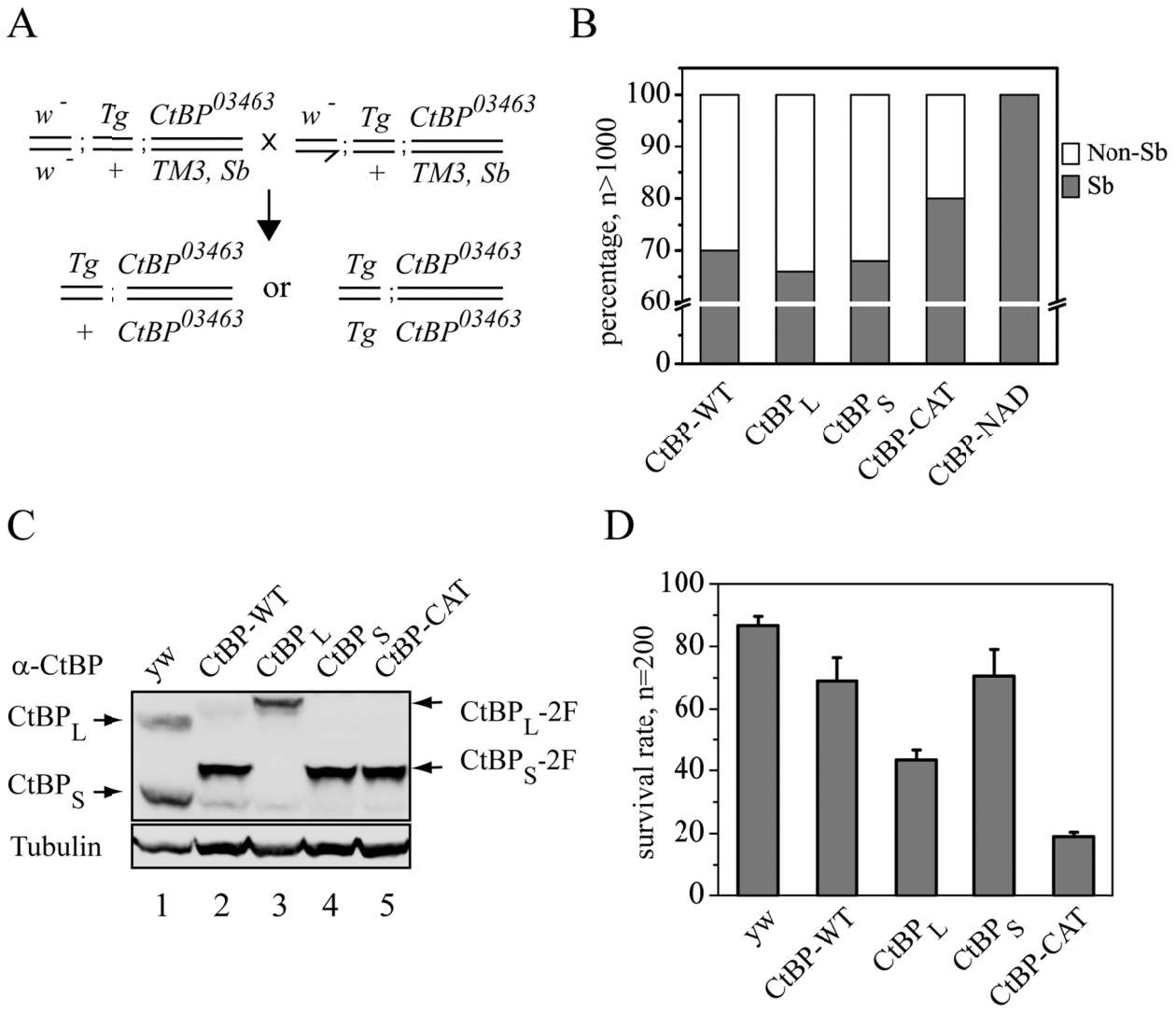

Figure 6. *CtBP$_L$*, *CtBP$_S$*, and *CtBP-CAT*, but not *CtBP-NAD*, rescue a *CtBP* mutant.

Figure 6 (cont'd)

Figure 6. *CtBP*$_L$, *CtBP*$_S$, and *CtBP-CAT*, but not *CtBP-NAD*, rescue a *CtBP* mutant. (A) Final cross used to assay zygotic rescue of *CtBP*^{03463} mutants. Tg, transgene. (B) *CtBP* transgenes rescue *CtBP*^{03463} lethality to adulthood. Percentage of *CtBP*^{03463} heterozygous and rescued progeny adult flies from final crosses (white portion of bars). More than 1,000 flies were counted for each rescue cross. The percentages of rescued adult flies from *CtBP-WT*, *CtBP*$_L$ and *CtBP*$_S$ crosses fit the theoretical prediction of 33%, while the *CtBP-CAT* rescue results in a lower percentage (20%; $P<0.0001$; z-test), indicating only a partial rescue by *CtBP-CAT*. No rescue was observed with the *CtBP-NAD* mutant; the only progeny from these crosses contained one chromosome containing a wild-type endogenous copy of *CtBP* (marked with *Sb*). (C) Expression of Flag-tagged CtBP proteins in rescued adult flies. Rescued flies from each cross were subjected to Western blot analysis with anti-CtBP antibody. Lane 1, endogenous CtBP proteins in non-transgenic flies. Lanes 2-5, the slower migrating Flag-tagged forms of CtBP were observed in all rescued flies. A weak band migrating at the size of endogenous CtBP$_S$, most likely a proteolytic product, was also occasionally seen. The rescue of a different *CtBP* null allele indicates that endogenous CtBP protein is not required for rescue (see text for details). (D) Maternal rescue capacity of CtBP isoforms and catalytic mutant judged by embryonic survival. Rescued flies were crossed *inter se* to eliminate contribution of maternal CtBP and embryo viability was assayed. *CtBP*$_L$ and *CtBP-CAT* rescued embryos have considerable lower hatch rates than *CtBP-WT* and *CtBP*$_S$ rescues ($P<0.0001$; z-test).

transgene (P<0.0001; z-test), while no complementation at all was observed for the *CtBP-NAD* transgene (Figure 6B).

To verify that these rescued flies were indeed homozygous for the *CtBP* mutation, extracts from adults were analyzed by Western blot with anti-CtBP antibody. The Flag-tagged forms of CtBP protein, which migrate somewhat slower than the endogenous proteins, were observed in all rescued adult flies. A weak band migrating at the size of endogenous $CtBP_S$ was also seen in some cases (Figure 6C, lanes 2 and 3), which may be a degradation product of the transgene or low levels of the endogenous protein. The absence of this product in preparations from many individual *CtBP*-rescue flies suggests that this is a proteolytic product of the recombinant protein, and low to nonexistent levels of endogenous CtBP are present in these flies (data not shown). To test whether low levels of endogenous CtBP were contributing to rescue, we repeated the rescue with $CtBP^{87De\text{-}10}$ mutant allele (a null or stronger hypomorphic allele) which was placed over one of two *CtBP* deletions (Poortinga et al., 1998; Stern et al., 2007). The same rescue results were obtained, indicating that the rescue activity of these transgenes is not allele-specific, but can be observed in different *CtBP* mutant backgrounds (crosses described in Materials and Methods; data not shown).

To eliminate the maternal CtBP contribution and dissect functions of CtBP variants more stringently, the rescued adult flies were crossed *inter se* so that the females, and consequently the oocytes, also contained no endogenous CtBP protein, and embryo viabilities were assayed. As shown in Figure 6D, the hatch rates of embryos rescued with the *CtBP-WT* and $CtBP_S$ transgenes were 69% and 70%, respectively, similar to that of wild-type embryos (*yw*, 87%), while only 43% and 19% of those rescued with the $CtBP_L$ and *CtBP-CAT* transgenes survived (P<0.0001; z-test),

demonstrating a significantly weaker ability to rescue. Introducing one copy of the $CtBP_L$ transgene into the $CtBP_S$ background did not increase the hatch rate, suggesting that this combination of the two proteins did not provide a more potent activity (data not shown).

Wing phenotypes of *CtBP* rescued flies. *CtBP-WT*, $CtBP_L$, $CtBP_S$, and *CtBP-CAT* rescued $CtBP^{03463}$ lethality, allowing development to proceed to adulthood, but their abilities to provide endogenous CtBP function varied. Apart from embryonic lethality tested above, the main observable phenotype was a marked effect on wing development. A variety of wing defects were observed in zygotic rescued adults, ranging from mildly curly wings to blistered wing phenotypes, and in some cases severely reduced wings (Figure 7A, ii-v). A spectrum of phenotypes was observed in all rescue assays; however, the $CtBP_L$ rescued adults exhibited significantly stronger wing phenotypes than did the *CtBP-WT* and $CtBP_S$ lines (Figure 7B, C). The *CtBP-CAT* mutant showed an even higher penetrance and consistently exhibited the most severe phenotypes, particularly when its partial lethality was taken into account (Figure 6B, Figure 7B, and C). Scanning electron microscopy images of normal and abnormal wings showed that the organization of wing epithelial cells was disrupted (Figure 7A, viii), supporting the idea that *CtBP* may regulate epithelial gene expression (Grooteclaes et al., 2003). The same wing phenotypes and trends were observed in rescued flies that had been bred for several generations so that there was no maternal contribution of endogenous CtBP, and the sole source of CtBP protein was the transgene (data not shown). Interestingly, although the *CtBP-NAD* mutant failed to rescue and was thus not testable in a *CtBP* mutant background, when placed in a genetic background containing only one copy of the endogenous *CtBP* gene, it induced a notched wing

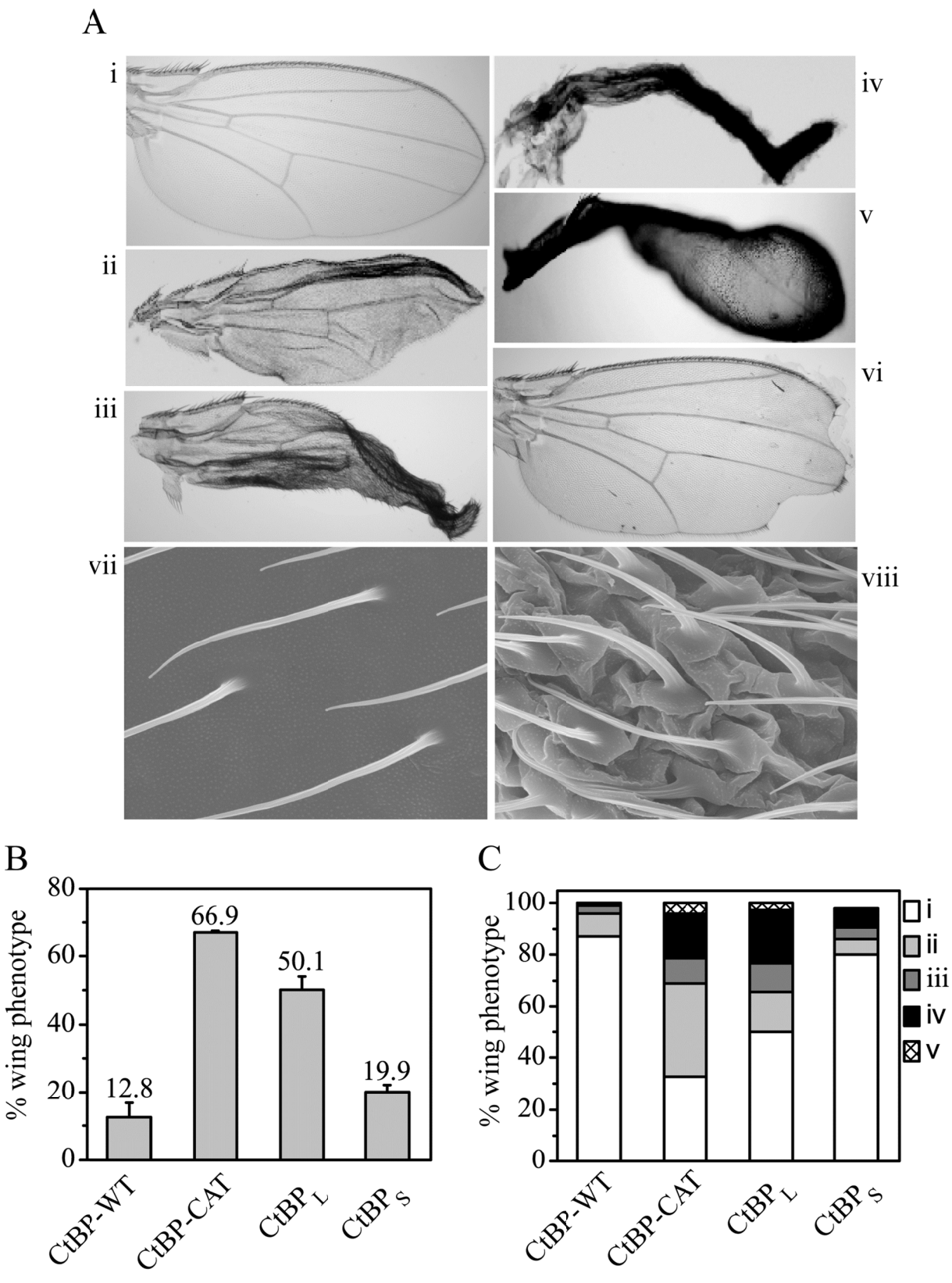

Figure 7. Wing phenotypes of *CtBP* rescued flies.

Figure 7 (cont'd)

Figure 7. Wing phenotypes of *CtBP* rescued flies. (A) Spectrum of abnormal wing phenotypes observed in rescued adult flies (ii to v). Representative defective wing phenotypes observed with *CtBP-WT*, $CtBP_L$, $CtBP_S$, and *CtBP-CAT* rescued flies (e.g. *Tg*/+; $CtBP^{03463}/CtBP^{03463}$). A normal wing is shown in i. vi. Notched wings observed specifically in *CtBP-NAD* transgenic individuals (*CtBP-NAD/CtBP-NAD*; $CtBP^{03463}$/+). Scanning electron microscope images of normal (vii) and abnormal (viii) wings reveal disruption of wing epithelial cell structure in a *CtBP-CAT* rescue fly (class iii). Similar disruption was also observed in class iv and v at high magnification. (B) Percentage of *CtBP* rescued flies exhibiting wing phenotypes of any degree of severity. *CtBP-CAT* and $CtBP_L$ rescue lines exhibited a higher percentage of wing phenotypes (P<0.0001; z-test). (C) Distribution of normal (i) and abnormal (class ii to v) wings in each rescue. *CtBP-CAT* and $CtBP_L$ rescued flies exhibit stronger wing phenotypes.

phenotype (Figure 7A, vi). This phenotype appeared in a dosage dependent manner; 34% of *CtBP* heterozygous mutant flies carrying two copies of *CtBP-NAD* showed notched wings, while no phenotype was observed when these transgenes were present in a wild-type background, or if only one copy of *CtBP-NAD* was present.

Bristle phenotypes of *CtBP* rescued flies. Severe depletion of CtBP has been observed to affect bristle development in the adult, through the corepressor's involvement in *E(spl)*-mediated gene expression in sensory neurons (Poortinga et al., 1998; Stern et al., 2007; Stern et al., 2009). Consistent with these reports, we also observed missing and extra macrochaete bristle phenotypes in *CtBP* rescued flies, especially with *CtBP-CAT* and $CtBP_L$ lines (data not shown). Thus, the macroscopic morphological defects associated with the partially functional *CtBP* alleles are not limited to wing development.

Gene expression alterations in *CtBP* rescued flies. CtBP functions primarily as a transcriptional corepressor, therefore we speculated that the impaired embryonic viability and wing phenotypes we observed in *CtBP* rescued embryos and adult flies may be associated with defects of its repression activity. *even-skipped* (*eve*) is a CtBP target whose expression pattern is disrupted in a *CtBP* mutant (Nibu et al., 1998a; Poortinga et al., 1998). We examined the embryonic *eve* expression pattern in *CtBP* rescue lines and observed that about 15% of *CtBP-WT*, $CtBP_L$, and $CtBP_S$ rescued embryos showed fusion of *eve* strips 2/3 and 4-6 (Figure 8A, ii, and 6B), similar to aberrant *eve* expression in a *Krüppel* (*Kr*) mutant (Nibu et al., 1998a). This phenotype is considerably elevated in *CtBP-CAT* rescued embryos (35%), and *CtBP-NAD* embryos, which are not rescued, were even more severely affected (45%), indicating CtBP activity was affected more by the catalytic and NAD(H) binding mutations. Considering that the *CtBP-NAD* failed to rescue and thus the embryos we examined were a mixture of transgenic and

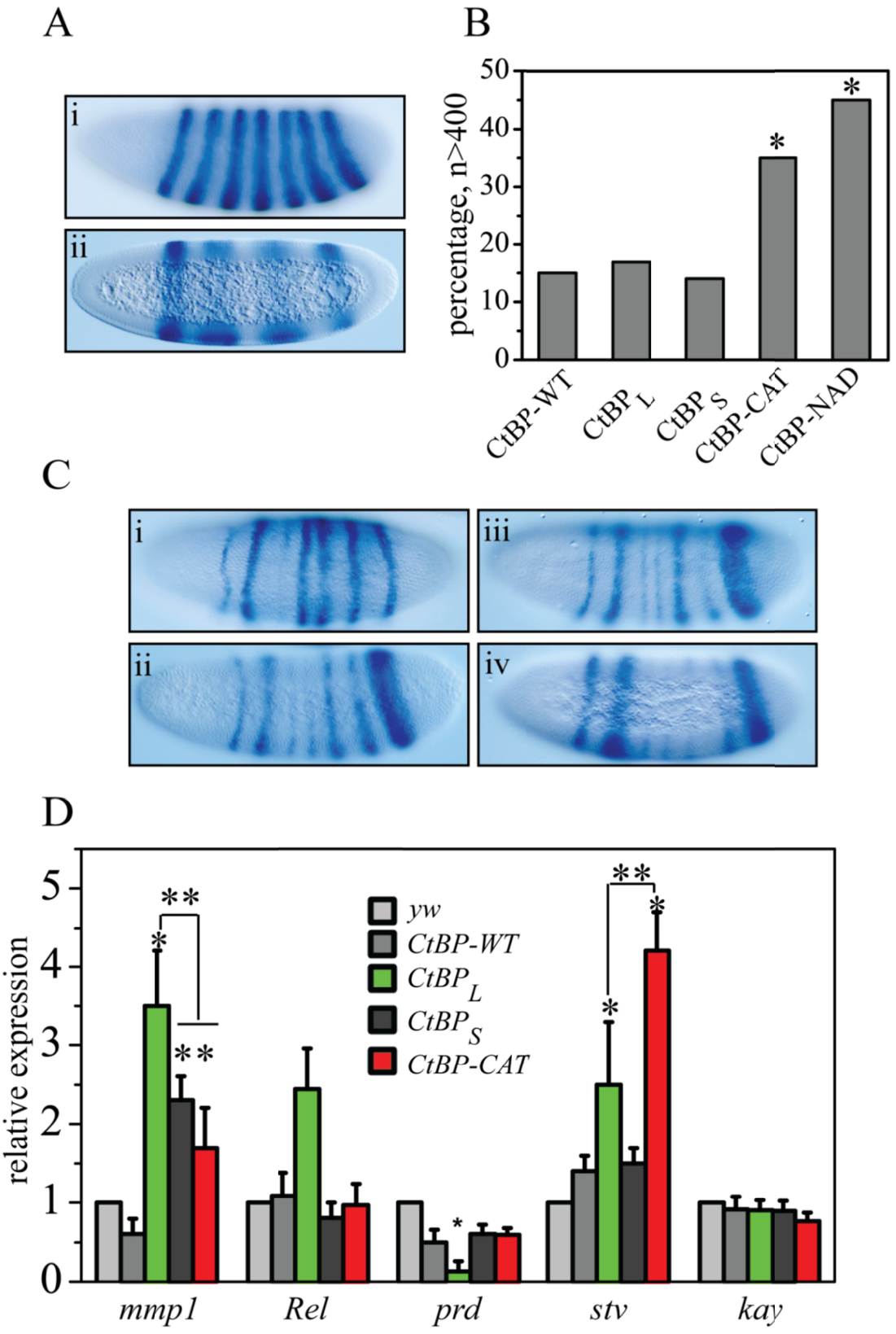

Figure 8. Gene expression alteration in *CtBP* rescue embryos.

Figure 8 (cont'd)

Figure 8. Gene expression alteration in *CtBP* rescue embryos. (A) *eve* expression patterns in *CtBP* rescue embryos. i. Normal *eve* pattern showing seven stripes. ii. Fusion of *eve* stripes 2-3 and 4-6. Image shows *CtBP-CAT* mutant embryos. This pattern was observed in all rescues; but most prominently in *CtBP-CAT* and *CtBP-NAD* lines. (B) Percentage of embryos showing disrupted *eve* patterns in different mutants. *CtBP-CAT* and *CtBP-NAD* mutant embryos showed significantly higher frequency of this abnormal pattern (* $P<0.0001$; z-test) (C) Abnormal *eve* patterns observed specifically in *CtBP-CAT* rescue embryos. Reduction of stripes 1/3 (i), 1/3/4 (ii), 3/4/6 (iii), and 3-6 (iv) were observed. (D) Gene expression alterations in *CtBP* rescued flies. Total RNA was extracted from pools of ten rescued adult flies (equal numbers of male and female) and mRNA levels were assessed by RT-qPCR.. *mmp1* and *Rel* expression was upregulated, with the highest change observed in the $CtBP_L$ rescue. *stv* levels were significantly elevated in $CtBP_L$ and *CtBP-CAT* rescues. *prd* expression level was significantly down-regulated in the $CtBP_L$ rescue background. *kay*, another gene bound by CtBP (3), did not show changes in any *CtBP* mutant. *β-Tubulin-56D* was used as reference gene for normalization. Bars represent means ± SE calculated across three independent biological replicate experiments. * $P < 0.001$, ** $P < 0.01$ by Student's t test.

mutant forms, the percentage is likely to be underestimated. Specifically in *CtBP-CAT* rescued embryos, however, we observed a novel pattern of *eve* disruption, showing reduction of stripes 3-6 (Figure 8C), which is reminiscent of *eve* patterns in a *knirps* (*kni*) mutant, suggesting the *Kr* and *kni* repression activities may be affected by the catalytic mutant (Nibu et al., 1998a).

To test if genes in other stages are affected by different forms of CtBP, we performed quantitative PCR to measure transcripts of selected genes that are bound *in vivo* by CtBP (Bianchi-Frias et al., 2004; Celniker et al., 2009). In adult flies, we found that expression of the *mmp1*, a potential CtBP target that has CtBP bound around the promoter, was increased more than three-fold in $CtBP_L$ lines (Figure 8D), with somewhat smaller effects in *CtBP-CAT* and $CtBP_S$ lines. *Rel*, another potential CtBP target, was induced more than 2-fold specifically in $CtBP_L$ mutants. The *starvin* (*stv*) gene was upregulated four-fold in the *CtBP-CAT* mutant, with a smaller but still significant upregulation in the $CtBP_L$ line (Figure 8D). Expression in *CtBP-WT* and $CtBP_S$ lines was minimally affected. These effects were reproducibly observed in multiple biological experiments, indicating the C-terminus and dehydrogenase activity are involved in CtBP-mediated repression in this context. For these genes, we observed loss of repression, consistent with loss of corepressor function in CtBP-CAT and $CtBP_L$ backgrounds; however, another CtBP target, *prd*, showed reduced expression in the $CtBP_L$ background, suggesting that there may be context-specific effects that allow CtBP to function in a stimulatory manner in some cases.

Discussion

CtBP is recruited by diverse transcription factors to effect repression of target genes in numerous metazoan regulatory pathways. Reflecting its evolutionary relatedness to dehydrogenases, CtBP has a functional NAD(H) binding cleft integral for overall protein structure, as well as for interaction with transcription factors and cofactors. In addition, CtBP contains active site residues that are both evolutionarily conserved and confer *in vitro* dehydrogenase activity. In this study, we utilized an *in vivo* developmental assay to address key questions about the function of CtBP. In a genomic rescue assay that comprehensively tests biological function, we show that the residues required for enzymatic activity as well as the C-terminal regulatory domain are essential for normal function in development (Figure 7, 6). Interestingly, animals programmed solely with the catalytic mutant protein or the form containing the C-terminal regulatory extension had significantly impaired viability and strong wing phenotypes. Specific transcriptional defects were also evident; embryonic *eve* expression was severely disrupted in these mutant embryos and expression levels of particular targets in adults were derepressed (Figure 8). The overall phenotypes of these mutants were for the most part enhanced manifestations of effects noted with less penetrant alleles, suggesting that loss of enzymatic function, or too extensive provision of the C-terminal extension reduced CtBP activity. The effects noted here are likely to represent a significant loss of activity, because CtBP function exhibits a considerable degree robustness: the *CtBP* gene is recessive, showing no phenotype with ~50% of normal dosage, and wild type flies carrying extra copies of these transgenes (up to ~200% of normal CtBP levels) are unaffected. Despite the lack of overt phenotypes for catalytic or C-terminal variants when expressed in cell-based assays, our analysis clearly indicates that the catalytic activity and C-terminal extension are important features that regulate CtBP function.

The results illuminate overall functional understanding of CtBP, leading to a model that explains the role of NAD(H) binding and catalysis (Figure 9). Previous results demonstrated that NAD(H) binding is important for folding of CtBP, and influences dimerization, binding of cofactors, and interaction with transcription factors (Balasubramanian et al., 2003; Kumar et al., 2002; Kuppuswamy et al., 2008; Nardini et al., 2009; Verger et al., 2006; Zhang et al., 2002; Zhao et al., 2009). NAD(H) has been suggested to do more than influence the structure of CtBP complexes, however. The observation that the CtBP-NAD mutants largely localize in the cytoplasm suggests that NAD(H) binding might also regulate protein subcellular localization (Figure 5F). In agreement with this hypothesis, mammalian CtBP1-NAD mutants also showed improper nuclear localization in MEF90 (*CtBP*$^{-/-}$) cells (Kuppuswamy et al., 2008). In addition, because of a reported higher affinity of the corepressor for the reduced NADH dinucleotide over NAD^+, CtBP has been suggested to serve as a mediator that links cellular redox status to transcriptional output, and treatment of cells with agents that affect NADH levels can influence CtBP-mediated repression and occupancy of promoters (Garriga-Canut et al., 2006; Kim et al., 2005; Zhang et al., 2002; Zhang et al., 2007). Building on these insights, an essential additional question is how a putative dehydrogenase activity may function in transcriptional control. Here, we have little in the way of precedent to go on; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been implicated in transcriptional regulation as an essential component of the OCA-S/Oct-1 coactivator complex, where it plays a critical role in S phase activation of histone H2B expression (Zheng et al., 2003). This dehydrogenase is also proposed to function as a redox sensor, monitoring changes during S phase, but how dehydrogenase activity itself affects transcription in this case remains a mystery (Dai et al., 2008; Yu et al., 2009). With respect to CtBP, numerous cell-based studies have shown that the dehydrogenase defective mutant remains

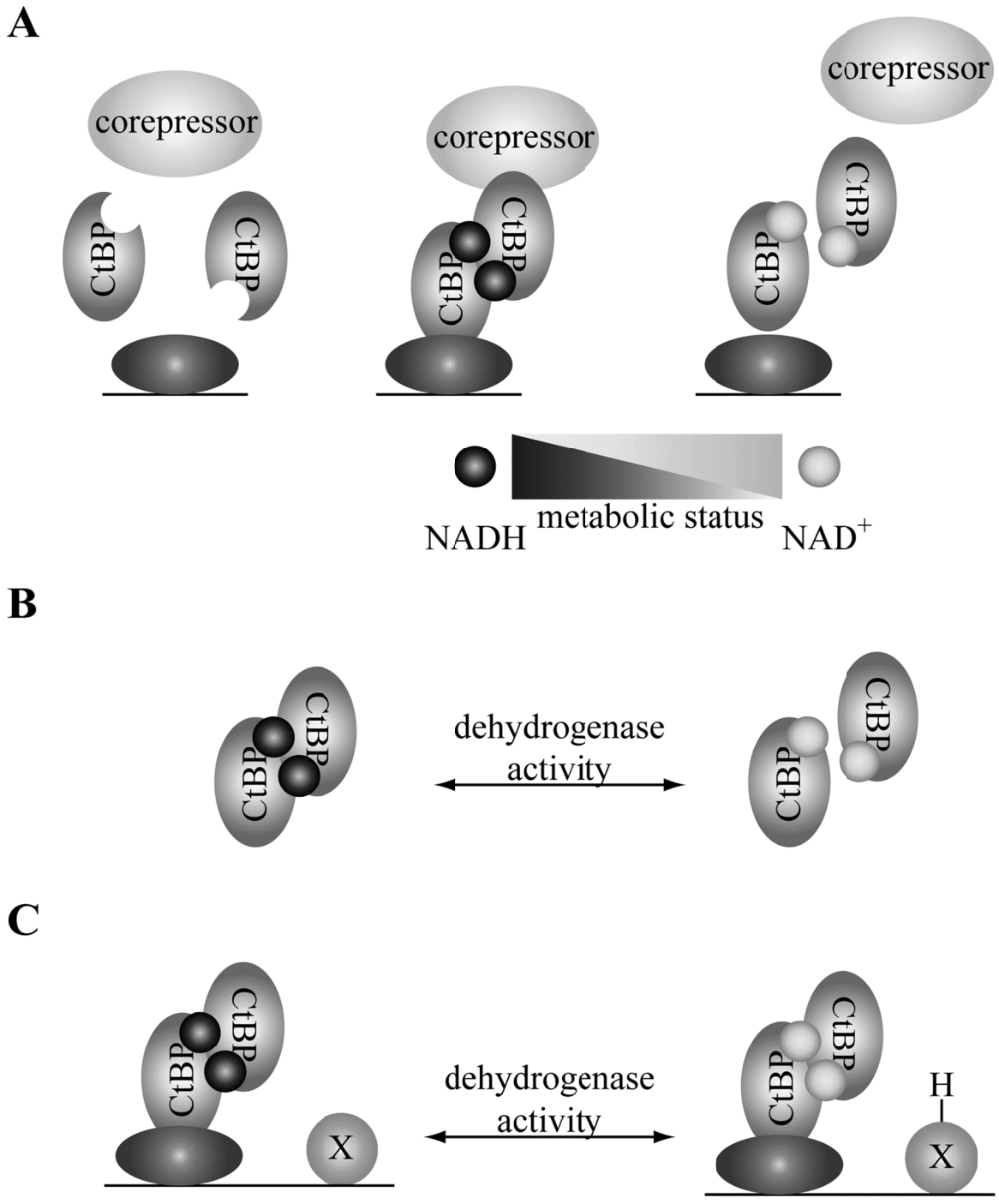

Figure 9. CtBP-directed integration of cellular metabolic status with gene expression, and possible roles for dehydrogenase activity.

Figure 9 (cont'd)

Figure 9. CtBP-directed integration of cellular metabolic status with gene expression, and possible roles for dehydrogenase activity. (A) Binding of NADH promotes dimerization, and interaction with DNA-bound transcription factors and cofactors. Interaction with NAD^+ permits weaker associations, and attenuates activity. Decreases in free cellular NADH levels thus reduce overall CtBP activity (38, 40). (B) The dehydrogenase activity may be mostly relevant to interconversion between the reduced and oxidized form of NAD to alter CtBP structure and hence activity. (C) Alternatively, reduction or oxidization by CtBP of a particular substrate critical for gene regulation may affect gene expression.

active, suggesting that the enzymatic activity is dispensable for CtBP function in these settings (Grooteclaes et al., 2003; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004). These previous studies were not designed to test whether CtBP dehydrogenase activity is important only under certain physiological conditions or on specific target genes, however. Our genomic rescue data strongly suggest that the dehydrogenase activity is important for proper development, and is involved in gene regulation. Two models would explain this function; possibly in response to metabolic signals, the enzymatic activity may be important as a mechanism to interconvert the reduced and oxidized forms of NAD, altering CtBP structure and thus interaction with cofactors, or the CtBP dehydrogenase activity may be important for reduction or oxidation of specific as-yet unknown substrates important for gene regulation (Figure 9). We cannot rule out the possibility that the mutation in the catalytic site may also affect CtBP interactions with a cofactor, but structural and mutational studies indicate that the catalytic site is located in a buried cleft, separate from the cofactor binding domain (Nardini et al., 2003).

An additional area of CtBP biology concerns possible regulation through the C-terminal region. Diverse metazoan CtBP proteins feature evolutionarily divergent C-terminal extensions that are dispensable for corepressor activity. Indeed, although *Drosophila* and other arthropods genes encode a conserved C-terminal region as an alternatively spliced exon, the major isoform lacks this domain (Mani-Telang and Arnosti, 2007). Previous studies have indicated that the C-terminal domain is subject to posttranslational modification through sumoylation and phosphorylation that may regulate CtBP through alternative cellular localization and degradation (Lin et al., 2003; Zhang et al., 2005; Zhang et al., 2003). Here we show that mutant animals that only express the $CtBP_L$ isoform with the C-terminal extension are viable, but exhibit impaired viability, defective wings and gene expression defects, clearly indicating the functional

importance of this domain (Figure 6D, Figure 7 B and C). It appears that equipping all CtBP molecules with this extension reduces CtBP activity, suggesting that this domain serves a negative regulatory function, possibly as a recipient of posttranslational modifications that may reflect signal transduction activity. In total, these results indicate that CtBP may function as a nexus of signal integration, responding to metabolic status through NAD(H) binding and signaling pathways through the C-terminus to affect transcriptional levels. An important question is whether this signaling would affect CtBP function generally, or in a gene-specific manner. The differential effects of CtBP-CAT mutant and $CtBP_L$ on *eve*, *mmp1, Rel, prd*, and *stv* (Figure 8C and D) provides some support for the latter possibility. A genome-wide comparison of expression profiles between *CtBP-WT* and *CtBP-CAT* as well as $CtBP_L$ mutants will provide important insights on this question.

In light of the involvement of CtBP in numerous regulatory events in *Drosophila*, including gap gene repression function in the blastoderm embryo, development of the peripheral nervous system, and recruitment in Notch, wingless and TGF-β signaling pathways, it was initially quite surprising that the prevalent phenotypes we observed in different *CtBP* rescues were specific for wings. However, further characterization of impaired embryo viability and disrupted *eve* expression in embryos, as well as bristle phenotypes and altered expression of specific genes in adult flies clearly shows that effects are not limited to wings. It is likely that changes in CtBP activity produce variable effects in different tissues, and those individuals with strongest effects in non-wing tissues do not survive.

In summary, we have demonstrated that the dehydrogenase activity of CtBP and the C-terminal domain are important for fine-tuning CtBP function in the context of development. A detailed biochemical characterization of how these activities are integrated remains to be

elucidated; important clues will likely come from a comprehensive picture of how possible modifications and metabolic signals are focused through CtBP to affect gene expression at a genomic level.

Acknowledgements

We thank K. Basler (University of Zurich, Zurich, Switzerland) for providing the pattB vector; W. D. Fakhouri, R. Sayal, and L. Zhang for assistance with confocal microscopy; I. Dworkin for fly wing imaging; the Bloomington stock center (Indiana) for fly stocks; anonymous reviewers for comments on the manuscript, and members of the Arnosti lab for useful discussions. This work was supported by NIH grant GM56976 to D.N.A.

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CHAPTER 3

CONSERVED CATALYTIC AND C-TERMINAL REGULATORY DOMAINS OF THE CTBP COREPRESSOR REGULATE OVERLAPPING AND UNIQUE CELLULAR PROCESSES

Abstract

The CtBP transcriptional corepressor regulates multiple cellular processes and plays critical roles in development and disease. CtBP is homologous to D2 hydroxy acid dehydrogenase enzymes, including conserved catalytic residues important for dehydrogenase activity, and possesses in addition a C-terminal regulatory domain. We previously showed that the active site and C-terminal regulatory domain of CtBP play important roles in diverse developmental settings. Here we use microarray analysis to comprehensively identify genes misregulated in flies solely expressing forms of CtBP that lack a key catalytic residue or the C-terminal regulatory domain. We find that misregulated genes are enriched in common and unique cellular pathways, further supporting the role that the putative dehydrogenase activity and the C-terminal domain play in transcriptional regulation. In particular, we show that the CtBP may modulate innate immune gene expression in *Drosophila*.

Introduction

Transcriptional regulation in development requires precise control of temporal- and spatial-gene expression by transcriptional activation and repression. Although sequence-specific DNA-binding transcriptional repressors determine the specificity of corepressor action, the transcriptional corepressors can provide diverse activities, and are themselves subject to multiple levels of regulation. Corepressors often form complexes with chromatin modifiers, such as histone deacetylases, histone (de)methylases, and nucleosome remodelers, proteins which alter chromatin structure when recruited to target genes to silence gene expression. The importance of corepressors in repression has been appreciated at cellular levels; however their biological functions in development of multicellular organisms are less well understood.

The C-terminal binding protein (CtBP) is a conserved transcriptional corepressor that plays important roles in development and diseases (Chinnadurai, 2002, 2009). Loss of *CtBP* in *Drosophila* and mice causes embryonic lethality (Hildebrand and Soriano, 2002; Nibu et al., 1998; Poortinga et al., 1998). The specific gene targets of CtBP provide clues to molecular mechanisms by which CtBP controls developmental processes in normal and disease states; CtBP promotes epithelial-to-mesenchymal transition (EMT) and cancer cell migration by repressing epithelial genes such as *E-cadherin* (Grooteclaes et al., 2003; Zhang et al., 2006), and it can influence cell cycle and signaling through repression of tumor suppressor genes including *PTEN* and $p16^{INK4A}$ (Grooteclaes et al., 2003; Mroz et al., 2008). CtBP lacks a DNA-binding domain, thus it is recruited to target promoters to repress gene expression through interactions with sequence-specific transcription factors. Indeed, a conserved CtBP-binding motif (PXDLS), which was first found as a motif facilitating binding of the adenoviral protein E1A to CtBP, was found subsequently in numerous other transcription factors that are implicated in multiple

cellular processes (Chinnadurai, 2002; Schaeper et al., 1995; Turner and Crossley, 2001). A number of studies linked CtBP to chromatin deacetylation, but the detailed repression mechanism mediated by CtBP was largely unknown, until the purification and characterization of a CtBP complex (Shi et al., 2003). Shi and colleagues purified the mammalian CtBP complex from tissue culture cells and showed that it contains multiple histone modifying enzymes, specifically histone deacetylases HADC1/2, histone methyltransferases, and NPAO, which was characterized later as the first histone demethylase, LSD1 (Shi et al., 2004; Shi et al., 2003). These findings indicate that that CtBP may repress gene expression through altering chromatin structure.

Interestingly, CtBP shows striking sequence similarities to NAD(H)-dependent D-2-hydroxyacid dehydrogenases (Schaeper et al., 1995). Consistent with its bacterial counterparts, CtBP also contains a highly conserved NAD(H)-binding motif (Rossmann fold) and a putative active site, a conserved His-Glu-Arg triad, in which the histidine is essential for catalytic activity (Chinnadurai, 2002; Kumar et al., 2002). Biochemical studies proved that CtBP is indeed an NAD(H)-dependent dehydrogenase and exhibits weak activity *in vitro* (Kumar et al., 2002). Although the physiological substrate of CtBP dehydrogenase activity has not been identified definitively, 2-keto-4-methylthiobutyrate, an intermediate in the methionine salvage pathway, has been identified as the best substrate tested to date (Achouri et al., 2007). This metabolite can inhibit CtBP repression activity *in vivo* and is specifically cytotoxic to cancer cells, raising the possibility of targeting CtBP for cancer therapy (Straza et al., 2010). However, the importance of the intrinsic dehydrogenase enzymatic activity in CtBP-mediated transcriptional regulation is largely unknown. The NAD(H)-binding activity of CtBP, in contrast, is clearly critical for CtBP repression activity. Disrupting NAD(H)-binding affects CtBP dimerization (Kuppuswamy et al.,

2008; Nardini et al., 2009; Thio et al., 2004), association with transcription factors (Kumar et al., 2002; Zhang et al., 2002), and cellular localization (Kuppuswamy et al., 2008; Zhang and Arnosti, 2011). CtBP NAD(H)-binding mutants also fail to repress reporter gene expression *in vivo* (Sutrias-Grau and Arnosti, 2004), and are not able rescue a *CtBP* homozygous lethal mutant (Zhang and Arnosti, 2011), demonstrating the essential roles of NAD(H) binding in CtBP function. The conformational change induced by NAD(H) when bound to CtBP may be one mechanism of how NAD(H) binding may regulate CtBP function (Kumar et al., 2002; Nardini et al., 2003). Surprisingly, however, structural and biochemical evidence showed that CtBP preferentially binds to NADH over NAD^+, with >100 fold higher affinity for NADH than NAD^+ (Fjeld et al., 2003; Nardini et al., 2003; Zhang et al., 2002), suggesting CtBP may function as a cellular redox sensor that is highly sensitive to changing levels of the reduced dinucleotide. In line with this hypothesis, studies have shown that treatment of cells with agents altering cellular NADH/NAD^+ ratio influenced CtBP occupancy at target promoters and repression outcome (Garriga-Canut et al., 2006; Kim et al., 2005; Zhang et al., 2002; Zhang et al., 2006; Zhang et al., 2007).

Mammalian CtBP proteins all contain an intrinsically unstructured C-terminal domain (Chinnadurai, 2002; Nardini et al., 2006). Although it is not clear yet what precise roles this domain may play in CtBP function, a regulatory function has been suggested by studies showing that multiple posttranslational modifications occur in this region. For example, phosphorylation of CtBP at Ser422 by HIK2 triggers proteome-mediated degradation (Zhang et al., 2005; Zhang et al., 2003), while sumoylation of Lys428 profoundly affected its cellular localization (Lin et al., 2003). Interestingly, however, a splicing variant of CtBP lacking this C-terminal domain has been identified in *Drosophila* and was shown to be the major isoform during *Drosophila*

development (Mani-Telang and Arnosti, 2007). The two major isoforms found in *Drosophila*, namely $CtBP_L$ and $CtBP_S$, so called because of the presence and absence of the C-terminal domain, otherwise identical, are produced by alternative RNA splicing (Poortinga et al., 1998; Sutrias-Grau and Arnosti, 2004). $CtBP_L$ and $CtBP_S$ are expressed throughout *Drosophila* development, are conserved in insects, and exhibit differential developmental regulation, indicating they may possess unique and overlapping functions (Mani-Telang and Arnosti, 2007). However, the distinct functions of these two isoforms are still a mystery. When tethered directly to DNA, these two isoforms showed similar repression activity in a reporter assay (Sutrias-Grau and Arnosti, 2004).

Building on these insights, we previously have tested the importance of the structural features of CtBP, namely the NAD(H) binding activity, the dehydrogenase activity, and the C-terminal domain, in a developmental context using a whole animal rescue assay (Zhang and Arnosti, 2011). We showed that the NAD(H) binding activity is essential for CtBP function, and we provided strong evidence for the first time that the intrinsic enzymatic activity, as well as the C-terminal domain, are critical for CtBP function in diverse developmental settings. To further understand the molecular functions of these important structural features, we analyzed the transcriptome profiles of mutant animals expressing either the catalytic-inactive form of CtBP (CtBP-CAT), or the isoforms with ($CtBP_L$) or without ($CtBP_S$) the C-terminal domain, in the absence of endogenous CtBP protein. Our results show that the conserved catalytic residue and the C-terminal domain are essential for correct transcriptional regulation of numerous genes, some of which are differentially affected by only one of these features of the protein. The

genome-wide regulation revealed by gene expression in *CtBP* mutants indicates that this corepressor is involved in regulation of multiple cellular processes.

Materials and methods

Fly strains. Fly stocks were maintained at 25 °C. The *yw* strain was used as a control. *CtBP* rescue flies, namely *CtBP-WT, $CtBP_L$, $CtBP_S$, and CtBP-CAT* were generated as previously described (Zhang and Arnosti, 2011). All rescue flies are homozygous for a *CtBP* null allele $CtBP^{03463}$ (Nibu et al., 1998; Poortinga et al., 1998) and contain only rescue gene.

Microarray expression analysis. Total RNA from rescued adult flies was extracted using RNeasy Mini Kit (Qiagen) following manufacturer's instructions, including on-column DNase digestion step. Ten adult flies from each genotype (yw, *CtBP-WT, $CtBP_L$, $CtBP_S$, and CtBP-CAT*), five males and five females, were used for each extraction, and three biological replicates were prepared for each sample. RNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies), and RNA integrity was measured with the Agilent 2100 bioanalyzer (Agilent Technologies). RNA samples with $OD_{260}/OD_{280} > 1.8$, and little to no degradation were submitted for microarray analysis. Probe labeling (Cy5 for mutant sample, Cy3 for *yw* control), hybridization to Agilent *Drosophila* G2519F 44K arrays, scanning, and feature extraction were performed by the Research Technology Support Facility at Michigan State University. The arrays were scanned using the Agilent G2505B Array Scanner followed by data and QC analysis with Agilent Feature Extraction software.

Data normalization and statistical analysis. Background subtraction, normalization and $\log_2$ transformation of expression signals were performed according to the Agilent Feature

Extraction Two-Color protocol. The resultant data were further analyzed using GeneSpring GX Version 11 (Agilent Technologies). One out of three replicates for *CtBP-CAT* and $CtBP_S$ turned out to be an outlier by correlation analysis (data not shown) and thus was excluded for further analysis. The remaining two data points were analyzed and the common significant genes were considered as significantly differentially expressed genes in *CtBP-CAT* and $CtBP_S$ mutants. Probes with raw intensities less than 20% in any of the three samples were excluded for subsequent analysis. *P*-value was calculated by Student's t test with Benjamini Hochberg FDR correction. Genes with corrected *P*-value < 0.05 and fold change (FC) > 2 (mutant/control) were considered as significantly differentially expressed genes and analyzed with DAVID (Database for Annotation, Visualization and Integrated Discovery) (Dennis et al., 2003; Huang da et al., 2009) to identify enriched gene ontology terms. We only analyzed GOTERM_BP (Biological Processes) at levels 3 and 4 to identify enriched terms within each subgroup.

Real-Time PCR. To confirm microarray data, new RNA samples from each genotype were prepared as described above and gene expression levels were quantified using SYBR Green PCR Master mix (Applied Biosystems). One microgram of total RNA was *in vitro* transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with RNase inhibitor in 20 ul volume. cDNA was then diluted into 20ng/5ul and 20ng of total cDNA was used in each 20 ul real-time PCR reaction. The final concentration of each primer was fixed to 250nM. All PCR primers were designed using Primer Express, version 3 (Applied Biosystems). The primer pairs labeled as AttA/B/C can detect *attacin-A*, *B*, and *C*. Similarly, the primer pairs labeled as CecA1/2 detects transcripts of *Cecropin A1* and *Cecropin A2*. PCR reactions were conducted as described previously with a disassociation step included (Zhang and Arnosti, 2011). Candidate transcript levels in three biological replicates for each sample were measured and

relative gene expression was determined using the Relative Expression Software Tool (Pfaffl et al., 2002), with β-*tubulin56D* (Fang et al., 2006) as reference gene. Statistical significance analysis was performed using Student's t test.

Results

Transcriptome profiles of *CtBP* mutant animals. Using a whole animal rescue assay, we previously showed that the conserved dehydrogenase activity, previously thought to be dispensable for overall transcriptional control (Grooteclaes et al., 2003; Kumar et al., 2002; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004), and the C-terminal regulatory domain fine-tune transcriptional responses during development (Zhang and Arnosti, 2011). Mutant animals expressing the catalytic defective form of CtBP (CtBP-CAT) or the isoform containing the C-terminal regulatory domain ($CtBP_L$) exhibited impaired embryonic viability, defective wing phenotypes, and disrupted target gene expression. Interestingly, a small-scale real-time PCR survey revealed a few targets that are distinctly affected by the catalytic activity and C-terminal domain, suggesting these two features may affect CtBP function in a gene-specific manner (Zhang and Arnosti, 2011).

To test this idea, we have examined the genome-wide expression profiles of the *CtBP* mutant adult animals that were rescued from lethality by expression of specific isoforms of the protein, namely, CtBP-CAT, $CtBP_L$, and $CtBP_S$, compared to wild-type (*yw*) control samples. A different sort of positive control sample, CtBP-WT, which carries a wild type version of the *CtBP* transgene in a *CtBP* null background, was also included. In flies rescued with the *CtBP-WT* transgene, only mild defects were noted in some of the mutant animals, and only 37 genes were misregulated (data not shown), indicating that the recombinant proteins are almost fully

functional in diverse developmental settings. To identify those genes that were specifically affected in *CtBP-CAT, $CtBP_L$*, and *$CtBP_S$* expressing lines, we focused on genes that were not misregulated in the *CtBP-WT* background. Compared to these controls, we identified 533 genes significantly (fold change >2) affected in *CtBP-CAT* animals, 824 genes in *$CtBP_L$*, and 569 genes in *$CtBP_S$* (Figure 10A). In the 824 misregulated genes in the *$CtBP_L$* line, 45% (374) genes were upregulated and 55% (450) were downregulated. Upregulated genes represented 34% (195) and 61% (327) in *$CtBP_S$* and *CtBP-CAT* backgrounds, respectively (Figure 10B). The larger number of significant changes in gene expression in the *$CtBP_L$* expressing flies indicates that the constitutive addition of the C-terminal domain has a more disruptive effect than the complete loss of this form of the protein (as is the case in the *$CtBP_S$* flies), suggesting that the $CtBP_S$ isoform may be a more potent repressor. These findings are consistent with the observations that CtBP can be negatively regulated through the C-terminal domain via posttranslational modifications (Lin et al., 2003; Zhang et al., 2005; Zhang et al., 2003). The high percentage of genes that are upregulated in the *CtBP-CAT* line suggests that this protein, lacking a catalytic site residue, is weakened as a corepressor, an activity that is important for the normal developmental activity (Zhang and Arnosti, 2011).

As shown in Figure 10A, there are overlaps between genes affected by the expression of *CtBP-CAT, $CtBP_L$* and *$CtBP_S$*, indicating that functions of CtBP related to catalytic activity and the C-terminal regulatory domain can impact common cellular pathways, directly and/or indirectly. A substantial number of misregulated genes were also uniquely affected by expression of particular isoforms, supporting our hypothesis that the enzymatic activity and C-terminal

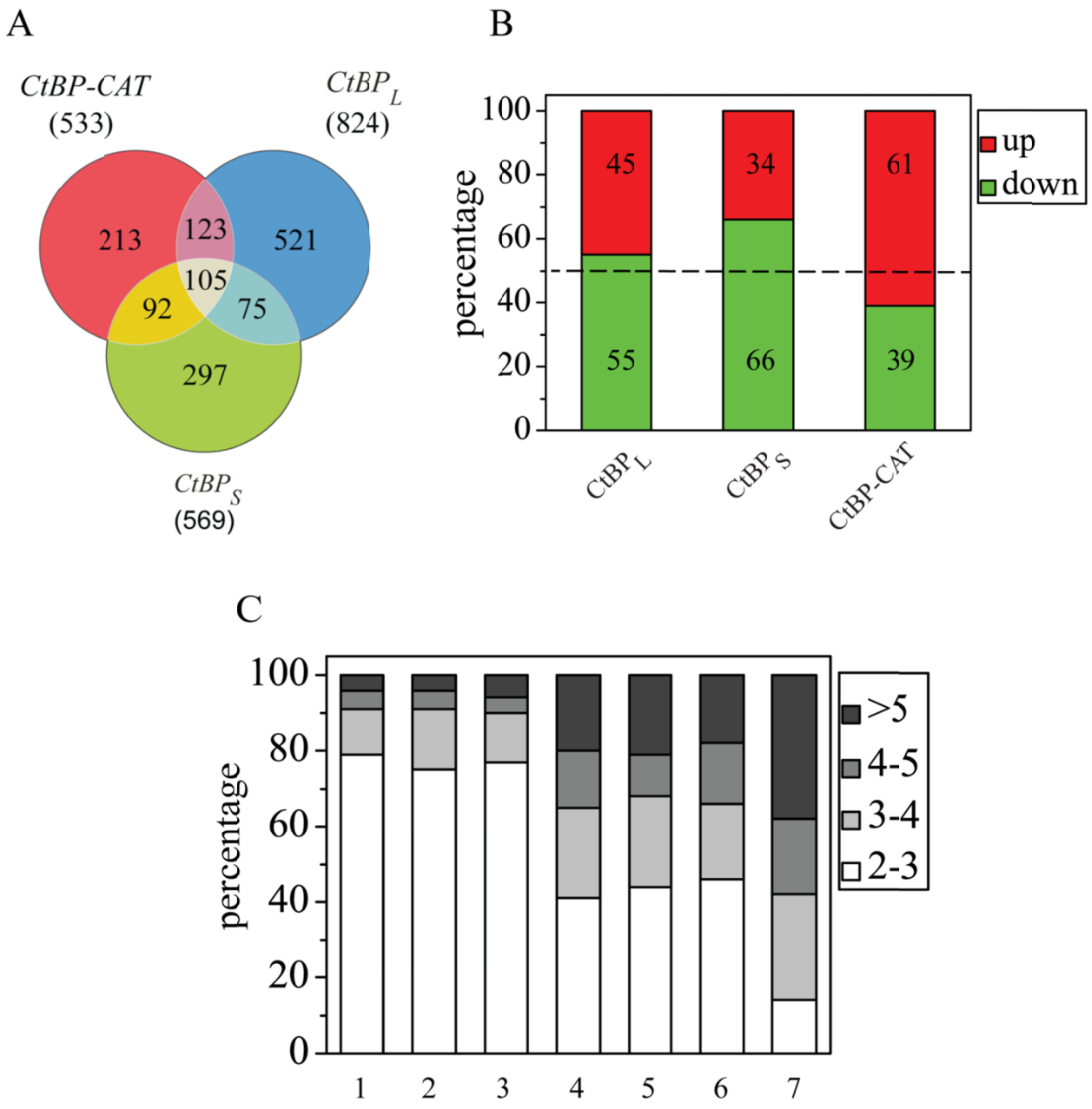

Figure 10. Transcriptome analysis of genes affected by unique expression of different forms of CtBP.

Figure 10 (cont'd)

Figure 10. Transcriptome analysis of genes affected by unique expression of different forms of CtBP. (A) Venn diagram shows genes significantly differently expressed in *CtBP-CAT* (533), $CtBP_L$ (824) and $CtBP_S$ (569) backgrounds. The number of genes affected by any two mutants, and by all three mutants is labeled accordingly. (B) Percentage of genes showing up- (red) or down-regulation (green) in $CtBP_L$, $CtBP_S$, and *CtBP-CAT* backgrounds. Dashed line indicates the position of 50%. (C) Percentage of genes falling into different fold-change ranges for individual and combinations of *CtBP-CAT,* $CtBP_L$, $CtBP_S$ lines. 1. *CtBP-CAT* specific targets (213); 2. $CtBP_L$ specific targets (521); 3. $CtBP_S$ specific targets (297); 4. Genes affected by expression of *CtBP-CAT* and $CtBP_L$(123); 5. Genes affected by expression of $CtBP_L$ and $CtBP_S$ (75); 6. Genes affected by expression of *CtBP-CAT* and $CtBP_S$; 7. Genes affected by expression of *CtBP-CAT,* $CtBP_L$, and $CtBP_S$.

domain may have unique, gene-specific functions. The majority of these uniquely affected genes showed only mild misregulation (less than threefold change, Figure 10C, lanes 1, 2, and 3; white bar). The percentage of genes exhibiting strong misregulation (greater than fivefold change, dark gray bar) is substantially greater among genes shared between any two mutant background (20%, compared to 5% in feature specific genes; lanes 4, 5, and 6), and was 40% in genes affected in all three mutants (lane 7). This trend may be explained by statistical effects, in that genes highly sensitive to changes in CtBP activity are more easily detected in microarray measurements, implying that some genes scored as uniquely affected by expression of *CtBP-CAT* would have also shown up in $CtBP_S$ or $CtBP_L$, but just missed the cutoff threshold for significant changes. Alternatively, those genes that are influenced by both the catalytic activity of CtBP as well as its C-terminal domain are most effectively repressed by the protein, leading to strong upregulation in the microarray experiments.

Regulation of distinct groups of genes associated with conserved catalytic and C-terminal regulatory domains of CtBP. The different numbers of misregulated genes, and different percentages of up- and down-regulated genes in different *CtBP* backgrounds led us to consider whether particular functional classes of genes are similarly affected by expression of particular CtBP isoforms. To identify possible functional enrichments of genes, the differentially expressed genes found in each mutant background were analyzed using a gene ontology statistical tool DAVID (Dennis et al., 2003; Huang da et al., 2009). The genes misregulated in *CtBP-CAT,* $CtBP_L$, and $CtBP_S$ backgrounds showed unique and common categories of enriched genes (Table 1). All three groups of genes were enriched for genes linked to innate immune/defense response and metabolic processes. Subsets of genes affected primarily by *CtBP-CAT*, however, were also enriched for signal transduction, ion transport, and metamorphosis.

Table 1. Enriched GO terms of feature specific targets and various overlaps.

Enriched terms	Count	Fold Enrichment	P-Value	Benjamini
CtBP-CAT				
innate immune response	12	4.4	7.90E-05	4.30E-02
signal transduction	36	1.7	1.70E-03	1.10E-01
aminoglycan metabolic process	14	3.3	2.90E-04	7.60E-02
ion transport	18	1.7	3.60E-02	6.30E-01
metamorphosis	18	1.6	5.20E-02	6.80E-01
$\boldsymbol{CtBP_L}$				
innate immune response	23	4.8	8.80E-10	1.30E-07
post-mating behavior	11	11.2	7.30E-09	5.60E-07
amine metabolic process	30	1.7	3.60E-03	1.20E-01
carbohydrate metabolic process	32	1.6	1.10E-02	2.80E-01
$\boldsymbol{CtBP_S}$				
defense response	15	2.9	5.70E-04	6.10E-02
amine metabolic process	20	2	4.20E-03	3.00E-01
carbohydrate metabolic process	22	1.9	5.90E-03	2.50E-01
cell redox homeostasis	7	4.5	4.40E-03	2.60E-01
CtBP-CAT **specific**				
neurogenesis	14	2.2	8.20E-03	8.30E-01
ion transport	11	2.5	1.00E-02	7.70E-01
metamorphosis	11	2.4	1.40E-02	7.80E-01
$\boldsymbol{CtBP_L}$ **specific**				
humoral immune response	12	4.4	6.80E-05	9.30E-03
reproductive behavior	12	4.2	1.00E-04	8.60E-03
$\boldsymbol{CtBP_S}$ **specific**				
NE				

Genes affected by *CtBP-CAT*, $CtBP_L$, and $CtBP_S$ were analysis by DAVID. Enriched Gene Ontology terms under Biological Processes at level 3 and level 4 were shown. Similar terms were combined for simplicity. Non-overlapping genes (Figure 10A) were analyzed similarly and enriched terms were shown. $CtBP_S$ specific targets did not reveal any statistically significant enrichment (P<0.05, Fold Change>2). NE, No Enrichment.

Similarly, a subset of genes affected by $CtBP_L$ was enriched for post-mating behavior, and genes affected by $CtBP_S$ included those enriched for cellular redox homeostasis (Table 1). The same analysis was further applied to each subgroup of genes, namely, *CtBP-CAT* specific genes, $CtBP_L$ specific genes, $CtBP_S$ specific genes (Figure 10A, non-overlapping subgroups). This analysis revealed that the *CtBP-CAT* specifically affected genes were enriched for neurogenesis, and $CtBP_L$ uniquely regulated targets were enriched for humoral immune response and reproductive behavior, while no statistically significant term enrichment was found in the $CtBP_S$ specific subgroup (Table 1; $P<0.05$). Interestingly, most of the neurogenesis and immune genes affected by *CtBP-CAT* and $CtBP_L$ were upregulated (Table 2), indicating that loss of the catalytic activity and the constitutive addition of the C-terminal domain may compromise CtBP's repression activity. In line with this observation, Garriag-Canut et al. reported recently that the neuron-restrictive silencer factor (NRSF, also known as REST), a master transcription factor in neurogenesis (Chong et al., 1995; Schoenherr and Anderson, 1995), interacts with CtBP and that its repression activity is regulated by metabolism through CtBP (Garriga-Canut et al., 2006). Disruption of the catalytic activity of CtBP may compromise its redox sensor function, as proposed previously (Zhang and Arnosti, 2011), resulting in misregulation of neurogenesis through NRSF and/or other neuron-specific transcription factors.

CtBP regulates *Drosophila* innate immune gene expression. As a transcriptional corepressor, CtBP has been shown to associate with a wide variety of transcription factors and play roles in multiple cellular processes (Chinnadurai, 2007), but it has not been previously reported to associate with innate immune responses. The innate immune response is a conserved

Table 2. Genes enriched in neurogenesis and innate immune responses in CtBP-CAT and $CtBP_L$ specific subgroups.

Probe ID	FlyBase ID	CG	Gene name	Fold Change
***CtBP-CAT* specific enrichment: neurogenesis**				
A_09_P041696	FBGN0000313	CG1744	*chaoptin*	2.5
A_09_P078341	FBGN0026252	CG7935	*moleskin*	-2.1
A_09_P051176	FBGN0038554	CG31247	*tincar*	2.5
A_09_P043141	FBGN0001981	CG3758	*escargot*	2.5
A_09_P033236	FBGN0023129	CG3705	*astray*	2.2
A_09_P106965	FBGN0003513	CG6993	*spineless*	2.0
A_09_P031456	FBGN0016047	CG13207	*no mechanoreceptor potential A*	2.6
A_09_P042331	FBGN0000634	CG6588	*Fasciclin I*	2.2
A_09_P031211	FBGN0015777	CG9261	*nervana2*	3.0
A_09_P077606	FBGN0025631	CG4322	*moody*	2.0
A_09_P029346	FBGN0011758	CG5529	*BarH1*	2.3
A_09_P010711	FBGN0004569	CG4531	*argos*	2.1
A_09_P044171	FBGN0003118	CG17077	*pointed*	-2.1
A_09_P011781	FBGN0005677	CG4952	*Dashshund*	2.2
***$CtBP_L$* specific enrichment: humoral immune responses**				
A_09_P050691	FBGN0034407	CG10794	*Diptericin B*	4.3
A_09_P199300	FBGN0014018	CG11992	*Relish*	2.2
A_09_P064286	FBGN0041581	CG18372	*Attacin-B*	3.8
A_09_P064281	FBGN0041579	CG4740	*Attacin-C*	2.6
A_09_P076236	FBGN0038530	CG7629	*Attacin-D*	3.4
A_09_P041556	FBGN0000250	CG5848	*cactus*	2.0
A_09_P041271	FBGN0000094	CG1361	*Andropin*	-2.6
A_09_P112895	FBGN0034539	CG11159	*CG11159*	2.8
A_09_P041636	FBGN0000279	CG1373	*Cecropin C*	5.2
A_09_P067231	FBGN0029765	CG16756	*CG16756*	2.0
A_09_P009876	FBGN0003882	CG10520	*tube*	-3.1
A_09_P054291	FBGN0035976	CG4432	*Peptidoglycan recognition protein LC*	2.1

Genes enriched in neurogenesis and innate immunity were revealed by GO analysis. Gene information and expression changes in particular mutant backgrounds are listed. Negative values represent down-regulation. Only one probe ID for each gene was shown.

process in *Drosophila* and human, and mainly regulated by the Toll and Imd pathways, which recognize different infections and activate expression of antimicrobial peptides via NF-κB transcription factors (Brennan and Anderson, 2004; De Gregorio et al., 2002). To further study the link between immune genes and CtBP, we compared our microarray data to a list of 134 immune-related genes identified in *Drosophila* by microarrays (De Gregorio et al., 2001). We found that 60 of these genes were misregulated in at least one of our *CtBP* backgrounds; these included *PGRP-SA*, *spatzle*, *PGRP-LC*, components of the Toll and Imd signaling pathways; *Relish* (a Rel-domain transcription factor, a homolog of NF-κB) and antimicrobial peptides (Table 3). Interestingly, most of these genes were misregulated in flies solely expressing $CtBP_L$ (95%, 57 out of 60), 50% of these genes were affected by *CtBP-CAT* (30 out of 60), but only 23% (14 out of 60) of them were affected by $CtBP_S$, indicating that the constitutive presence of the C-terminal domain has the strongest perturbation effect.We chose 25 well characterized immune-related genes (Obbard et al., 2009) which were also more directly linked to immune responses, including the ones mentioned above, extracted their expression signals from microarray and illustrated by a heatmap (Figure 11A).

To verify the microarray data, we designed primers for the 25 well characterized immune-related genes (Figure 11A) and re-evaluated their expression levels in newly prepared RNA samples with quantitative PCR. Among these genes, 13 of them were confirmed (primers labeled as *AttA/B/C* detect total levels of *attacin A, attacin B*, and *attacin C*; and *CecA1/2* detect both *Cecropin A1* and *Cecropin A2*). As shown in Figure 11B, most of these genes showed the biggest misregulation (except *ANP* and *Dro3*) in $CtBP_L$ background, confirming that constitutive presence of the C-terminal domain of CtBP has the strongest effect on expression of innate

Table 3. Immune related genes affected by $CtBP_L$, CtBP-CAT, and $CtBP_S$.

Flybase ID	Gene Name	Fold Change		
		$CtBP_L$	CtBP-CAT	$CtBP_S$
FBgn0033327	*PGRP-SC1b*	-3.1	-4.2	0
FBgn0035976	*PGRP-LC*	2.1	0	0
FBgn0035806	*PGRP-SD*	2.6	2.2	0
FBgn0035977	*PGRP-LF*	3.4	2.6	0
FBgn0028430	*Hemese*	2.7	0	0
FBgn0030051	*spirit*	0	8.9	6.1
FBgn0003495	*spatzle*	2.9	0	2
FBgn0003882	*tube*	-3.1	0	0
FBgn0000250	*cact*	2	0	0
FBgn0014018	*Relish*	2.2	0	0
FBgn0000094	*Andropin*	-2.6	0	0
FBgn0012042	*Attacin-A*	5	7.2	3.4
FBgn0041581	*Attacin-B*	3.9	0	0
FBgn0041579	*Attacin-C*	2.6	0	0
FBgn0038530	*Attacin-D*	3.4	0	0
FBgn0052279	*dro2*	0	5.4	0
FBgn0052283	*dro3*	4.1	0	0
FBgn0052282	*dro4*	3	4.3	4.7
FBgn0035434	*dro5*	0	0	2.8
FBgn0000277	*Cecropin A1; Cecropin A2*	4.6	6	0
FBgn0000279	*Cecropin C*	5.2	0	0
FBgn0004240	*Diptericin*	4.2	6.3	0
FBgn0034407	*DptB*	4.3	0	0
FBgn0014865	*Metchnikowin*	6	6.9	0
FBgn0035964	*Dhpr*	2.5	4.2	4.4
FBgn0020416	*Idgf1*	2.4	2.4	0
FBgn0243512	*puc*	2.1	0	0
FBgn0000422	*Ddc*	2.1	0	0
FBgn0034329	*IM1*	0	3.5	0
FBgn0025583	*IM2*	2.2	3.1	0
FBgn0033835	*IM10*	5.9	6.1	0
FBgn0034328	*IM23*	3.1	4.9	0
FBgn0010358	*δTry*	-2.2	-2.1	0
FBgn0011555	*θTry*	-2.2	0	3.6
FBgn0039778	*Jon99Fi*	-6.7	8.3	0
FBgn0031654	*Jon25Bii*	-2.6	-3.8	0
FBgn0031653	*Jon25Biii*	-3.1	-4.3	0
FBgn0035667	*Jon65Ai*	-3	-3.8	0
FBgn0001285	*Jon44E*	-2.4	-2.6	0

Table 3 (cont'd)

FBgn0023541	*Cyp4d14*	-3.3	-2.1	0
FBgn0010383	*Cyp18a1*	-2.8	0	0
FBgn0035790	*Cyp316a1*	-2.6	0	-2.2
FBgn0025454	*Cyp6g1*	2	0	0
FBgn0031693	*Cyp4ac1*	2.5	0	0
FBgn0031689	*Cyp28d1*	8.2	0	0
FBgn0002563	*Lsp1β*	-2.3	0	0
FBgn0005626	*ple*	3.6	0	0
FBgn0260746	*Ect3*	-6.6	0	-2.7
FBgn0034094	*Tsf3*	2	0	0
FBgn0037724	*Fst*	2.2	2.6	0
FBgn0044810	*Turandot X*	2.5	0	6.7
FBgn0000358	*Cp19*	3	0	0
FBgn0036023	*CG18179*	-2.5	3.5	0
FBgn0029765	*CG16756*	2	0	0
FBgn0039629	*CG11842*	2.6	3.3	2.3
FBgn0034539	*CG11159*	2.8	0	0
FBgn0046999	*CG6429*	3	0	0
FBgn0040582	*CG5791*	3.7	6.4	5.9
FBgn0030774	*CG9675*	4.9	4.8	6.6
FBgn0027584	*CG4757*	7.7	8	2.2

Information about immune-related genes misregulated in at least one of the three CtBP mutant backgrounds is listed. Negative values represent down-regulation. 0 means gene expression was not significantly affected ($P<0.05$, Fold Change>2).

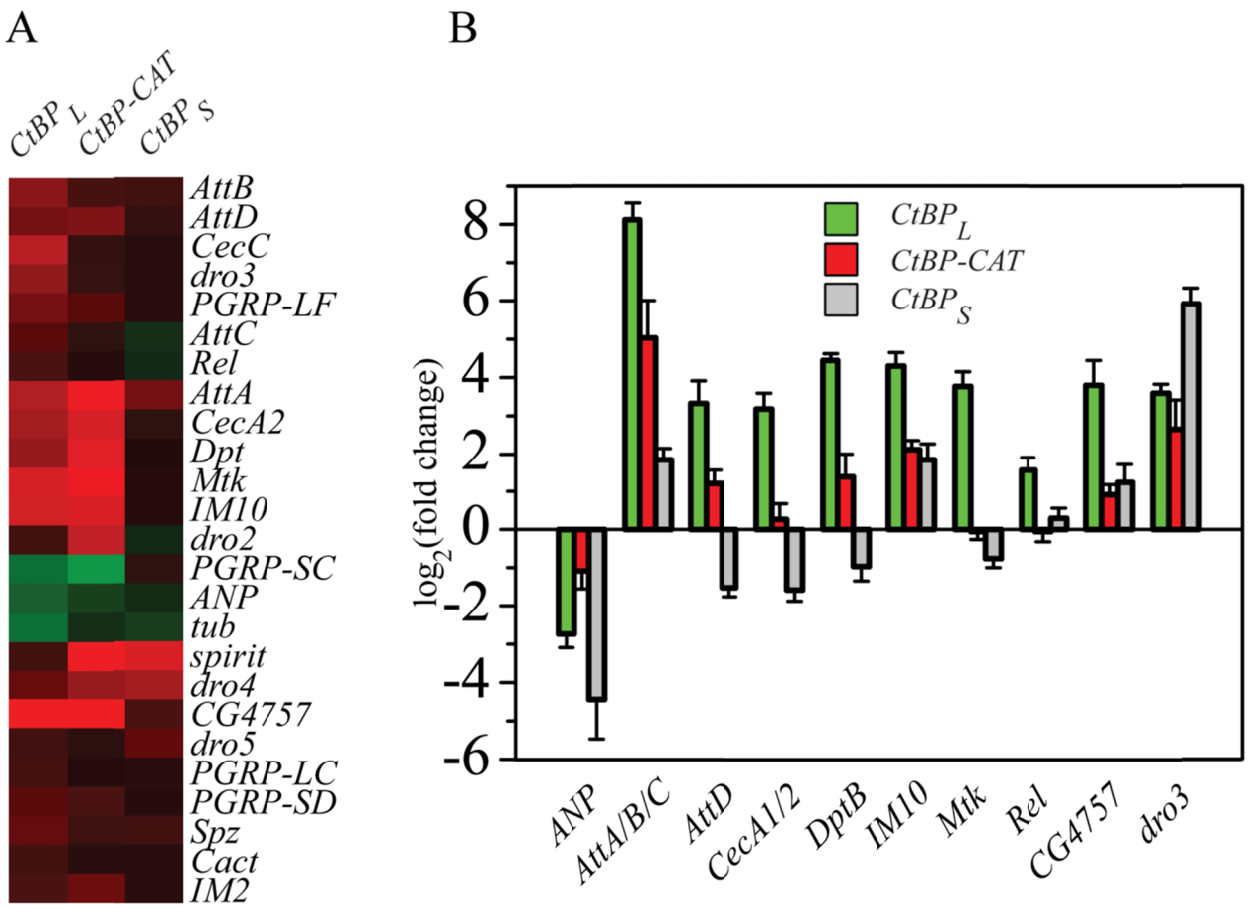

Figure 11. The catalytic activity and C-terminal domain of CtBP are important for regulation of regulate innate immune responses.

Figure 11 (cont'd)

Figure 11. The catalytic activity and C-terminal domain of CtBP are important for regulation of innate immune responses. (A) Expression levels of twenty-five well-characterized immune-related genes in *$CtBP_L$*, *CtBP-CAT*, and *$CtBP_S$* mutant backgrounds. Transcript levels, showing significantly differential expression or not, were extracted from microarray data and illustrated in a heatmap. Red indicates up-regulation, green depicts down-regulation. Scale: -4.2 – 8.9. Gene names are labeled on the right. (B) QPCR validation. New RNA samples were prepared and transcript levels of immune-related genes (Figure 10A) were measured quantitatively. Thirteen out of twenty-five genes were confirmed to be misregulated in $CtBP_L$ and/or CtBP-CAT mutants. *AttA/B/C* indicates total transcripts levels of *attacin A, B,* and *C* detected by one primer pair. *CecA1/2* shows total levels of *Cecropin A1* and *Cecropin A2* detected by a single primer pair. *β-Tubulin-56D* was used as the reference gene for normalization. Bars represent mean results ± standard errors calculated across three independent biological replicate experiments.

immune genes. To further test whether CtBP directly regulates the expression of the Immune-related genes, we knocked down CtBP in *Drosophila* S2 cells using dsRNA and measured their transcript levels. Although CtBP protein levels were reduced by 70%, the mRNA levels of the 25 immune-related genes (Figure 11A) were essentially the same (data not shown), There are several possible reasons why we did not see any change of gene expression in this system; it is possible that the overall reduction in CtBP activity is more profound in the backgrounds we tested than in a simple reduction of overall wild-type protein. Alternatively, the induction of these genes may require signals that are lacking in the cell culture system; simple loss of CtBP regulation may be necessary but not sufficient to up-regulate gene expression. Finally, general perturbations in global gene expression, or specific up-regulation of key mediators of innate immunity, may induce generalized stress responses in the adults that triggers coordinate upregulation of many immune-related transcripts. These signals may be lacking in the dissociated cell culture system.

Discussion

CtBP is an essential transcriptional corepressor that plays important roles in numerous metazoan regulatory pathways. Similar to other corepressors, such as Sin3 and Mi-2, which form corepressor complexes with histone-modifying enzymes, CtBP has been suggested to function as a scaffold to recruit histone-modifying activities to target promoters to suppress gene expression. Interestingly, however, CtBP also contains an intrinsic dehydrogenase activity. The possible significance of this enzymatic activity has been a long-standing question in the CtBP field. The evolutionary conservation of the dehydrogenase domain strongly indicates biological significance, but numerous studies have shown that this enzymatic activity is dispensable (Chen

et al., 2009; Grooteclaes et al., 2003; Mani-Telang et al., 2007; Sutrias-Grau and Arnosti, 2004). Another less understood area of CtBP biology is the C-terminal domain. Although this region is present in all mammalian CtBP proteins and a conserved isoform in *Drosophila*, and posttranslational modifications occurring in this domain have been identified and suggested to regulate CtBP properties, it is not required for CtBP's repression activity. Using a whole animal rescue assay, we have recently shown that the catalytic and C-terminal domain are important for modulating CtBP function in diverse developmental settings (Zhang and Arnosti, 2011). In this study, we took a genome-wide approach to identify downstream targets of CtBP that are affected by the catalytic activity and the C-terminal regulatory domain to gain insights into biological processes regulated by these structural features. We identified a total of 1,426 genes that are significantly affected by the loss of the conserved catalytic residue and/or the constitutive presence of the C-terminal domain in genomic rescue animals (Figure 10A), further supporting the idea that these two structural features are functionally relevant.

Consistent with the essential roles that CtBP plays in development, we found the GO term metamorphosis/imaginal disc development was significantly enriched (Table 1). Genes include *argos* (wing/eye-antennal disc morphogenesis), *blistery* (wing morphogenesis), *dachshund* (eye development), *pointed* (organ development), and others (data not shown). Genes with GO terms related to metabolism are also significantly over-represented. In agreement with the observation that the $CtBP_L$ and *CtBP-CAT* mutant flies are less reproductively fit (data not shown), we found that genes regulating reproductive behavior, such as accessory gland peptide *36DE/70A/33A*, and accessory gland-specific peptides (*Acp26Aa, Acp29AB*, and *Acp98AB*) are misregulated.

Our results point to a potential role for CtBP in innate immune responses. The molecular similarities of host defense between *Drosophila* and humans make it a valuable model organism

to study animal innate immunity. The *Drosophila* innate immune responses are mainly activated by two distinct signaling pathways, the Toll and Immune deficiency (Imd) pathways, which recognize fungal/Gram-positive bacterial and Gram-negative bacterial infections, respectively (Hoffmann and Reichhart, 2002). Activation of these two signaling pathways results in NF-κB-like transcription factors-mediated induction of antimicrobial peptides (AMPs) which were then secreted into the hemolymph to kill invading pathogens (Hoffmann and Reichhart, 2002). Immune-related genes, ranging from membrane sensors (*PGRPs*), to signal transducing molecules (*spz*), transcription factor (*Rel*), and antimicrobial peptides (*AMPs*), are largely misregulated in $CtBP_L$ and *CtBP-CAT*, but not $CtBP_S$ flies (Figure 11 and Table 3), and we confirmed this observation by RT-PCR. The upregulation of most of these transcripts is consistent with loss of CtBP corepressor activity, but as discussed above, a generalized stress response may also involve indirect CtBP regulation. To determine whether CtBP may directly bind to and regulate target genes, we examined *in vivo* occupancy of CtBP from *Drosophila* embryos (http://intermine.modencode.org/release-18/objectDetails.do?id=211000586). We found 437 out of 1,426 genes have the corepressor bound within 5 kbp of the transcriptional start site and noted that some immune-related genes regulated by CtBP (*IM10, CecA2, tub, Anp,* and *Rel*) were included in the list. Other genes lacked observable CtBP binding in the embryo; this occupancy may be specific to particular developmental stages, or some targets may be indirectly regulated. In any event, it will be interesting to test whether regulation of innate immune responses represents a conserved CtBP regulatory function.

Another set of genes misexpressed in the *CtBP* backgrounds were linked to neurogenesis. The function of CtBP in neurogenesis is not well understood. Garriga-Canut et al. reported that CtBP interacts with the NRSF transcriptional repressor that is responsible for silencing of

neuronal genes in non-neuronal tissues, and that metabolic signals relevant to NRSF activity are channeled through CtBP (Garriga-Canut et al., 2006). Of genes functionally affected by our *CtBP* lines and directly occupied by the corepressor in the embryo, as assessed by ChIP-chip studies, the category of "neurogenesis-related" is significantly enriched (data not shown and Table 2). These genes were only found to be misregulated in *CtBP* catalytic, but not $CtBP_L$ or $CtBP_S$, mutant background, indicating the NAD(H)-dependent dehydrogenase activity may play important roles in neurogenesis. We searched for potential binding sites of CtBP recruiting proteins among these 437 potential targets using MEME (Bailey and Elkan, 1994) and one of the most over-represented motifs (Figure 12) matched the NeuroD binding motif (Seo et al., 2007), indicating CtBP may interact with NeuroD and contribute to its function. NeuroD is a basic helix-loop-helix (bHLH) transcription factor playing critical roles in neurogenesis, but has been suggested to contain transactivation activity (Lee et al., 1995; Naya et al., 1995). It is not clear whether NeuroD may function as a repressor in a context-dependent manner with CtBP, or CtBP may activate certain NeuroD targets, as suggested previously (Fang et al., 2006).

Acknowledgements

We thank Pankaj Acharya for assistance with MEME analysis, and members of the Arnosti lab for useful discussions. This work was supported by NIH grant GM56976 to D.N.A.

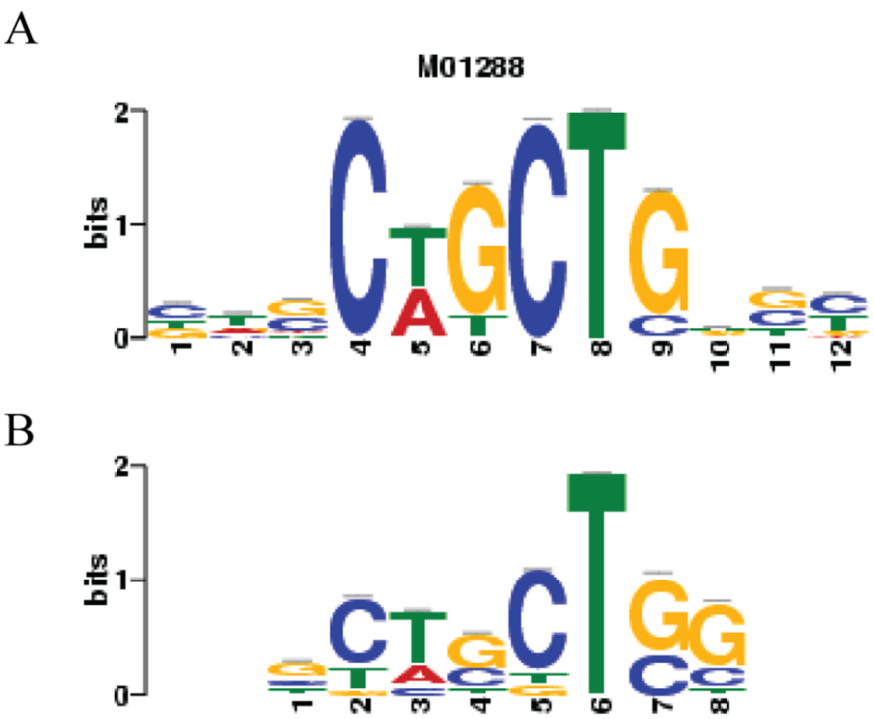

Figure 12. Over-represented NeuroD binding motif found among CtBP potential direct targets. *de novo* motif identification was performed using MEME; over-represented motifs were searched against motif databases via TOMTOM. (A) NeuroD binding motif found in transfac database. (B) Over-represented CtBP binding motif discovered by MEME (*P*-value: 5.7E-5, *E*-value: 0.12, *q*-value: 0.24).

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The extensive studies of the CtBP transcriptional corepressor in the past 15 years have greatly contributed to our understanding of transcriptional repression in development and disease. While genetic studies have firmly established the fundamental role of CtBP in animal development (Hildebrand and Soriano, 2002; Nibu et al., 1998; Poortinga et al., 1998), biochemical investigations have identified a large number of CtBP-associating transcription factors whose activities are dependent, although sometimes partially, on recruitment of CtBP, and hence illustrated the important roles of CtBP in a variety of cellular processes (Chinnadurai, 2002; Turner and Crossley, 2001). The studies of NAD(H)-binding activity of CtBP have revealed a unique and significant function of CtBP as a metabolic switch which is capable of regulating target gene expression through cellular metabolism, making CtBP an example of transcription factors that directly link metabolism and gene regulation (Fjeld et al., 2003; Zhang et al., 2002).

The biological relevance of the dehydrogenase activity of CtBP in transcriptional regulation, however, remains to be a mystery. The striking homology of CtBP to bacterial dehydrogenases and the absolute conservation of the catalytic residues strongly imply functional significance, but initial attempts testing the enzymatic activity in CtBP-mediated repression were unsuccessful. It is likely that such enzymatic activity begins to play a role in transcription only under certain conditions, and/or functions in a context-dependent manner, rather than is generally required. It is also possible that this dehydrogenase activity is important for other functions, in addition to transcriptional repression, of CtBP, which needs to be further investigated.

In this dissertation, we have tried to answer the long-standing question in the CtBP field: what is the biological significance of the enzymatic activity in CtBP function? Instead of testing the importance of this feature in repressing reporter genes in cell culture, as other studies have done, we took a whole-animal rescue strategy and asked if a catalytic inactive form of CtBP could rescue a CtBP homozygous lethal allele and restore development. The advantage of this whole animal rescue strategy over previous cell-based assays is that it allows us to test the importance of such enzymatic activity of CtBP in a developmental context, rather than under a single condition. Because we do not restrict the test to repression activity, other to-be-identified functions of CtBP which may require this enzymatic activity will also be tested in this assay. These functions may include Golgi membrane fission (Weigert et al., 1999), nervous system synapses (Schmitz et al., 2000), and cell division (Bergman et al., 2009; Spyer and Allday, 2006). Other roles suggested by our transcriptome and GO analysis described in Chapter 3 may include metabolism, cellular homeostasis, cellular redox homeostasis, and signal transduction. Furthermore, all the potential tests will occur under physiological conditions, a parameter that may affect protein function substantially. By generating mutant animals expressing solely the designed forms of mutant CtBP, we also eliminated a potential interference coming from the endogenous CtBP, which may mask the importance of the feature tested by forming a functional heterodimer with the exogenous mutant protein.

To summarize the findings, we provided strong evidence for the first time showing that the enzymatic activity of CtBP is indeed critical for the normal developmental activity of CtBP. although the catalytic inactive mutant rescued a CtBP lethal allele in general, a considerable percentage of mutant embryos died during embryogenesis. The survivors, however, were not absolutely normal either. A spectrum of defective wing phenotypes was observed in the

surviving mutant adult flies, emphasizing the relevance of this enzymatic activity during *Drosophila* development. Furthermore, we showed that the mutant embryos and adults exhibit defective target gene expression, directly linking the enzymatic activity to gene regulation.

In addition, we tested the biological relevance of the NAD(H)-binding activity and the C-terminal domain using the same strategy. Previous studies have revealed the importance of NAD(H)-binding in mediating CtBP and transcription factor interaction, and in CtBP repression, we further showed that the NAD(H)-binding is essential: a NAD(H)-binding mutant failed to rescue a CtBP lethal allele. The C-terminal domain has been suggested to play regulatory roles through post-translational modifications, but a non-tail containing form was still able to repress a reporter effectively. We showed in our study that this domain plays a role in proper development, as mutant animals expressing only the tail-containing form of CtBP exhibited impaired viability, defective wings, and gene expression defects.

To further understand the molecular functions of the enzymatic activity and the C-terminal domain, we identified genes misregulated in mutant animals expressing the catalytic inactive mutant form of CtBP, or the variants with or without the C-terminal domain. Our results revealed that the enzymatic activity and the C-terminal domain affect overlapping and unique targets. The results further confirmed that these two features of CtBP are truly important for gene regulation. GO analysis showed that non-overlapping targets are enriched in different biological processes, suggesting the enzymatic activity and the C-terminal may regulate distinct, as well as common, cellular pathways. Therefore, the study described in Chapter 3 may provide a line of evidence suggesting the idea that the enzymatic activity and the C-terminal domain of CtBP may be functionally separated. The fact that sumoylation and phosphorylation occurring on the C-terminal domain affect CtBP cellular localization and stability suggests that the C-terminal

domain may regulate CtBP overall function, rather than particular activities. In contrast, the catalytic activity may function in a context-dependent manner; a catalytic mutant form of CtBP seems to be able to restore the wide type expression profile.

The work described here provides strong evidence suggesting that the evolutionarily conserved enzymatic activity and the C-terminal regulatory domain are important for CtBP function in diverse developmental settings. This study revealed a previously under-appreciated *in vivo* function of such structural features of CtBP and is likely to help focus attention on investigating detailed molecular mechanisms of the enzymatic activity in gene regulation. Interesting questions immediately following from this study may include:

1. Identification of target genes directly and specifically regulated by the enzymatic activity and the C-terminal regulatory domain.

   The fact that the catalytic inactive mutant and the (non)tail-containing variants rescued the CtBP null lethality indicates that although the enzymatic activity and the C-terminal regulatory domain are important, they are not absolutely required for CtBP overall function. To dissect their molecular function, an immediate idea is to find their direct targets and study how these mutant forms affect target gene expression. We have tested this idea in Chapter 3 and identified hundreds of genes affected by these mutants in adult flies, but did not definitely identify their direct targets. By lowering the threshold from 2-fold to 1.5-fold, the non-overlapping genes did not merge together, but remain to be distinct groups, indicating they represent real feature-specific targets, rather than sensitivity artifacts (data not shown). One might combine our microarray data with CtBP Chromatin Immunoprecipitation followed by microarray (ChIP-chip) or direct sequencing (ChIP-Seq) to identify overlaps, which are likely to be feature-specific direct

targets. We did not map CtBP genome-wide bindings in adult flies, but rather made use of the currently available CtBP ChIP-chip dataset generated in embryos (link is provided in Chapter 2, Materials and Methods). We only found a small number of overlaps between these two datasets. It is possible that these overlapping genes are potential catalytic- and C-terminal domain-specific targets and worth further characterization. However, due to the different materials used (adult RNA for our microarray and embryo RNA for ChIP-chip), we may miss the real feature-specific targets resulting in CtBP dynamic binding during development. Considering the effective zygotic, but poor maternal, rescue, we speculate that these features may mainly function during embryogenesis. Therefore, identifying targets by microarray and mapping in vivo binding by ChIP-Seq using the same material across different time windows during embryogenesis hold better chances to find real direct feature-specific targets, which would pave the way for revealing molecular mechanisms of the enzymatic activity and C-terminal regulatory domain in CtBP-mediated gene regulation.

2. A second interesting question remaining to be elucidated is whether and/or how the enzymatic activity may link cellular metabolism to gene regulation. More and more examples have appeared showing that cellular redox status can modulate expression of CtBP target genes through differential binding of NADH and NAD^+ (Di et al., 2010; Kim et al., 2005; Zhang et al., 2002; Zhang et al., 2006; Zhang et al., 2007), but it is largely unknown if the enzymatic activity plays a role in this regulation. Testing if the catalytic inactive mutant would respond to cellular redox changes and modulate target gene expression in a similar manner could easily tell if the enzymatic activity is important in redox status sensing. For example, one might feed flies with agents such as $CoCl_2$, 2-DG,

or raise flies under hypoxic conditions, which have been shown to change cellular redox status. Notably, one might need to perform such experiments in the absence of endogenous CtBP to eliminate potential interference.

3. Another area of CtBP biology concerns its physiological substrates. Although certain small molecules such as pyruvate and 4-methylthio-2-oxobutyric acid (MTOB) can be catalyzed by CtBP dehydrogenase activity (Achouri et al., 2007; Kumar et al., 2002), the catalytic activity is very low compared with other dehydrogenases, indicating they are unlikely to be the *in vivo* substrates. Thus identification of the physiological substrates for CtBP dehydrogenase activity remains an open question and may reveal novel pathways applied by CtBP to link metabolism to gene regulation. Furthermore, a recent study has shown that the CtBP potential substrate, MTOB, can inhibit CtBP activity at high concentrations, and is more cytotoxic to cancer cells than normal cells (Straza et al., 2010), opening the possibility to target CtBP for therapeutic treatment of human cancer. Building on this insight, finding CtBP *in vivo* substrates may be of therapeutic beneficial.
4. CtBP functions, other than transcriptional repression, are also of much interest and importance. Previous studies have uncovered CtBP functions in Golgi membrane fission (Weigert et al., 1999) and synapse formation (Schmitz et al., 2000), but whether CtBP plays roles in other cellular processes is not well understood. Recent studies suggested that CtBP may play a role in cell division by associating with centrosomes and maintaining mitotic fidelity (Bergman et al., 2009; Spyer and Allday, 2006), providing another non-transcriptional function that CtBP may possess. A detailed phenotypic analysis of *CtBP* mutant animals, such as the $CtBP1^{-/-}$, $CtBP2^{-/-}$, and $CtBP1^{-/-}$ $CtBP2^{-/-}$ mutant mice, may be a good starting point to reveal additional roles that CtBP may play.

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