Review

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C-terminal binding proteins: central players in development and disease

Abstract: C-terminal binding proteins (CtBPs) were initially identified as binding partners for the E1A-transforming proteins. Although the invertebrate genome encodes one CtBP protein, two CtBPs (CtBP1 and CtBP2) are encoded by the vertebrate genome and perform both unique and duplicative functions. CtBP1 and CtBP2 are closely related and act as transcriptional corepressors when activated by nicotinamide adenine dinucleotide binding to their dehydrogenase domains. CtBPs exert transcriptional repression primarily *via* recruitment of a corepressor complex to DNA that consists of histone deacetylases (HDACs) and histone methyltransferases, although CtBPs can also repress transcription through HDAC-independent mechanisms. More recent studies have demonstrated a critical function for CtBPs in the transcriptional repression of pro-apoptotic genes such as Bax, Puma, Bik, and Noxa. Nonetheless, although recent efforts have characterized the essential involvement of CtBPs in promoting cellular survival, the dysregulation of CtBPs in both neurodegenerative disease and cancers remains to be fully elucidated.

Keywords: anoikis; apoptosis; cancer; C-terminal binding proteins; development; neurodegenerative disease.

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Introduction

C-terminal binding protein (CtBPs) were originally identified as binding partners for the adenovirus E1A protein, suggesting an important function for CtBPs in regulating cell survival (1, 2). CtBPs are evolutionarily conserved proteins and are present in a diverse number of species ranging from terrestrial plants to *Caenorhabditis elegans* to humans (3). Whereas the vertebrate genome encodes two highly homologous CtBP proteins, CtBP1 and CtBP2, the invertebrate genome encodes one CtBP transcript. The major splice forms of CtBP1 and CtBP2 function primarily as transcriptional corepressors. CtBPs associate with various DNA-binding repressors such as basic Krüppel-like factor 8 (BKLF8) to recruit chromatin-modifying enzymes to the promoter regions of DNA (4, 5). However, some minor CtBP splice variants also mediate various cytosolic functions (3).

Genetic knockout studies have demonstrated that CtBP1 and CtBP2 perform both unique and duplicative functions during development and genetic deletion of CtBPs results in severe developmental defects and embryonic lethality (6). Consistent with their role in transcriptional corepression, Grooteclaes and colleagues (4, 7) identified CtBPs as critical repressors of apoptotic and anoikis gene programs. Subsequent studies have demonstrated that CtBPs bind the viral oncoproteins EBNA3A and EBNA3C, further demonstrating that CtBPs have significant roles in regulation of cellular proliferation and survival (8, 9). Given the critical functions of CtBPs in promoting cell survival, dysregulation of CtBPs has emerged as a potential causative factor underlying neurodegenerative diseases as well as cancer.

In this review, we summarize current information on the structure, function, and regulation of CtBPs. Next, we discuss the involvement of CtBPs in development, apoptosis, and anoikis. Finally, we highlight recent data supporting dysregulation of CtBPs in neurodegenerative diseases and cancer. Given the extensive evidence demonstrating a pro-survival function for CtBPs in both non-neuronal and neuronal cell populations, CtBPs are emerging as novel molecular targets for the treatment of diseases characterized by either enhanced apoptosis (i.e.,

neurodegeneration) or increased resistance to apoptotic cell death (i.e., cancer).

Structure and function of CtBPs

Domain structure of CtBPs

CtBP1 and CtBP2 are highly conserved and share a significant degree of sequence and structural homology (Figure 1). Of these two proteins, CtBP1 has been the most extensively characterized. Although CtBP1 and CtBP2 possess high sequence homology and structural similarities, they have been shown to exert both unique and duplicative effects (6). Despite these diverse functions, however, all CtBP family members share several key characteristics that are essential for protein function.

A hydrophobic cleft known as the PXDLS-binding site is one of the best characterized features of the CtBP family and several CtBP-interacting partners containing PXDLS-binding motifs have been identified. In many cases, mutations in either the PXDLS-binding pocket in CtBPs or in the binding motif of interacting proteins is sufficient to abrogate their interaction (2, 10–14). Moreover, this hydrophobic cleft is essential to CtBP function because it plays an extensive role in recruiting other members of the CtBP corepressor complex in both a PXDLS-dependent and a PXDLS-independent manner (10). Specifically, the PXDLS-binding site is essential for recruiting the core machinery of the corepressor complex, including histone deacetylases (HDACs), histone methyltransferases (HMTases), and transcriptional repressors, which are required for CtBP to execute its corepressor functions (10). CtBPs can also bind to proteins directly *via* the PXDLS domain to repress their function (15).

In addition to the PXDLS-binding pocket, all CtBP family members also contain a surface groove that is capable of binding proteins with an RRT-binding motif (16). Like the PXDLS hydrophobic cleft, the RRT-binding pocket of CtBPs is predominantly used to bind and recruit members of the corepressor complex, and many CtBP-interacting proteins (CtIPs) that bind at this site also contain PXDLS-binding motifs (10, 16). Thus, each CtBP monomer contains two binding sites that can be occupied simultaneously by distinct members of the corepressor complex. Together, these two binding sites account for a majority of CtBP interactions with proteins of the corepressor complex and repressor proteins that recruit CtBPs to specific gene promoters.

Intriguingly, it is now well established that CtBP proteins share significant sequence homology with 2D hydroxyacid dehydrogenases, particularly within the RTT-binding cleft (Figure 1) (17). Moreover, the dehydrogenase domain contains a dinucleotide-binding site that is capable of binding both NAD+ and nicotinamide adenine dinucleotide (NADH) (17). Although CtBPs are functional dehydrogenases, the significance of this observation is not yet known, and the physiological substrate for CtBP deacetylase activity has not been identified (18). However, it has been established that the ability of CtBPs to bind NADH is required for these proteins to form dimers with one another and exert transcriptional repression (10, 18–20). Nonetheless, CtBP mutants that are deficient in their ability to form dimers do display the ability to mediate other CtBP functions, which are discussed in further detail below (10).

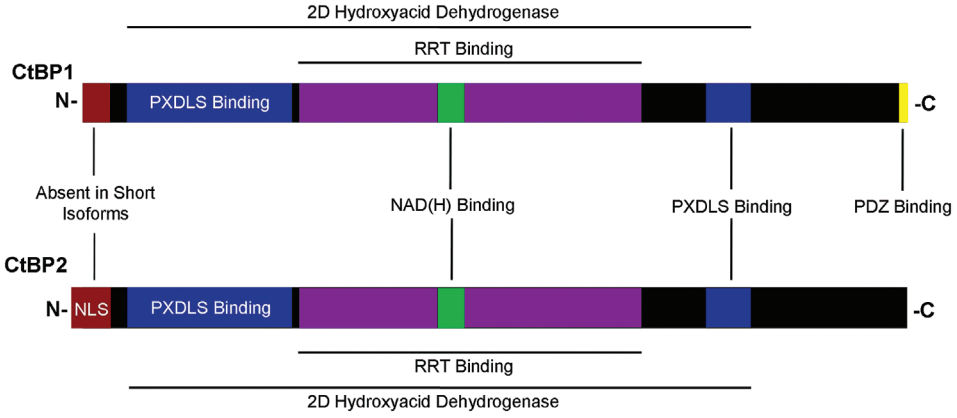

Figure 1 Structures of CtBP1 and CtBP2.
The PXDLS-binding cleft, RRT-binding cleft, and dehydrogenase domain of CtBP1 and CtBP2 compose the majority of the CtBP protein structure. Note the longer N-terminus of CtBP2, which contains an NLS that is absent from CtBP1. Also note that CtBP1 contains a PDZ domain at the C-terminus that is absent from CtBP2.

Collectively, the PXDLS-binding cleft, RRT-binding cleft, and dehydrogenase domain of CtBP1 and CtBP2 compose the majority of the CtBP protein structure and are highly homologous for both CtBP family members and their various isoforms (Figure 1). However, there are also slight differences in protein sequence that contribute to the differing functions of CtBP1 and CtBP2. Most notably, CtBP2 contains a nuclear localization signal (NLS) at its N-terminus that is absent in CtBP1 (21). This region of CtBP2 contains several lysine residues that are acetylated, contributing to the nuclear retention of this protein (22). The NLS is absent in some isoforms of CtBP2, resulting in the accumulation of a small cytoplasmic pool of this protein; however, the cytoplasmic function of CtBP2 remains unclear. In contrast, CtBP1 relies on binding to transcriptional repressors such as zinc finger E-box proteins (ZEB1/2), SUMOylation, and other interactions such as heterodimerization with CtBP2 for nuclear import and retention (21, 23, 24). Thus, CtBP1 can accumulate in the cytoplasm where it mediates membrane trafficking and Golgi fission during mitosis (25, 26). Moreover, CtBP1 possesses a PDZ-binding domain at its C-terminus that is not present in CtBP2, allowing CtBP1 to interact with proteins such as neuronal nitric oxide synthase (nNOS) to maintain its cytoplasmic localization (23). Other structural differences in CtBPs are the result of either alternative splicing or transcriptional initiation from alternative promoter regions that produce the various isoforms of CtBP1 and CtBP2.

CtBP corepressor activity

The primary function of CtBP family members is transcriptional repression. Because CtBPs do not possess intrinsic DNA-binding capabilities, these proteins are recruited to active transcription sites by DNA-binding transcriptional repressors such as ZEB1/2, Krüppel-like factors, Elk4, and E2F7 (14, 24, 27–29). Once bound to the repressor, CtBPs recruit histone-modifying enzymes to effectively halt transcriptional activity at sequence-specific gene promoters recognized by the transcriptional repressors (Figure 2). Among these enzymes are HDAC1/2, Ubc9, and the HDAC1/CoREST/lysine-specific demethylase 1 (LSD1) complex, which comprise the core machinery of the corepressor complex (10). There are also a number of proteins including an HMT complex comprised of G9a, WIZ, CDYL, and GLP, and three SUMO E3 ligases, HPC2, PIAS1, and Pc2 that act as auxiliary components in the corepressor complex, enhancing gene repression through histone modifications and acting as a stage for the SUMOylation of a number of CtIPs (10, 30).

Structurally, CtBPs are capable of binding members of the corepressor complex at both the hydrophobic

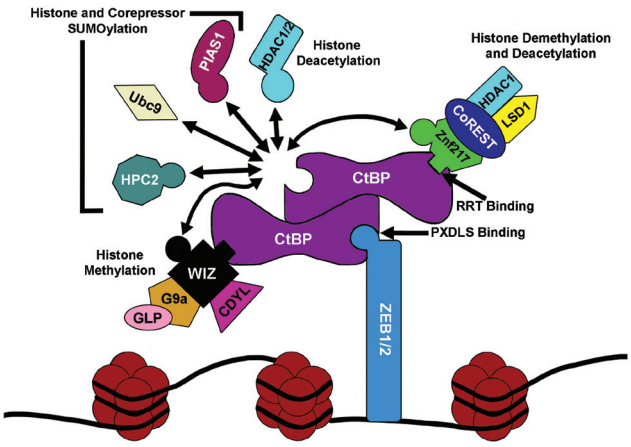

Figure 2 Transcriptional regulation by CtBPs.
As CtBPs do not bind to DNA directly, they recruit various chromatin-modifying enzymes to exert transcriptional repression. CtBPs can recruit a corepressor complex consisting of HDAC1/2, Ubc9, and the HDAC1/CoREST/LSD1 complex. CtBPs can also recruit an HMT complex comprised of G9a, WIZ, CDYL, and GLP, and the SUMO E3 ligases, HPC2 and PIAS1, that repress gene transcription through histone modifications and/or by acting as a stage for the SUMOylation of a number of CtIPs.

PXDLS-binding cleft and the RRT-binding cleft located near the dinucleotide-binding site for NADH (10, 16). Additionally, the PXDLS-binding site may bind multiple proteins at once, one containing a PXDLS-binding motif and others interacting through PXDLS-independent associations (10). Therefore, CtBPs may mediate a diverse number of histone remodeling reactions that ultimately result in transcriptional silencing of a target gene. Further, as NADH binding is essential for the formation of CtBP dimers, it is also necessary for the corepressor activity of these proteins. Moreover, NADH binding to CtBPs affects the overall stability of CtBP-binding interactions, enhancing the affinity of CtBPs for binding to interacting partners *via* the PXDLS-binding pocket (20).

A majority of the corepressor activity associated with CtBPs is mediated by the activity of HDACs, which may interact directly with CtBPs or be recruited as part of the HDAC1/CoREST/LSD1 corepressor complex (10). These enzymes deacetylate histone 3 at lysine 9, and this site is subsequently methylated by the activity of auxiliary HMTase components such as G9a (31). Subsequent to this reaction, LSD1, which depends on recruitment by Znf217 and CoREST to interact with CtBP1, is able to demethylate lysine 4 located on histone 3 (32). Notably, histone 4 has also been shown to be a target of SUMOylation, and this modification has been linked to transcriptional repression (33). Thus, there is also speculation that the SUMOylation machinery associated with the corepressor complex, including Pc2, Ubc9, HPC2, and PIAS1, also plays a role

in modifying histones to silence target gene expression. SUMOylation is also thought to modulate the activity of components of the corepressor complex to either enhance or restrict their function, thereby regulating the extent to which gene expression is repressed. This will be discussed in further detail in the following section.

CtBPs can also mediate transcriptional repression through a mechanism independent of the corepressor complex. This occurs by CtBPs directly binding and inhibiting the histone/factor acetyltransferase p300 and the transcriptional activator P/CAF (15, 34). Intriguingly, SUMOylation of p300 reverses its activity and causes it to act as a transcriptional repressor (35). Therefore, it is possible that recruitment of the SUMOylation machinery to this protein by interactions with CtBPs may also play a role in inhibiting the transactivating function of p300, contributing to transcriptional repression of target genes (36).

Derivation and function of CtBP isoforms

Both the *CtBP1* and *CtBP2* genes produce multiple isoforms. In addition to their nuclear functions, some of these isoforms play important roles within the cytoplasm that are unrelated to CtBP corepressor activity. For example, the *CtBP1* gene produces long and short isoforms known as CtBP1-L and CtBP1-S, respectively (37). Although both of these isoforms are capable of participating in transcriptional corepression at the nucleus, they also possess unique cytoplasmic functions. Indeed, CtBP1 has been found to localize to ribbon synapses in sensory neurons, where it is thought to provide structure to synaptic ribbons (38, 39). In addition, CtBP1-S, more commonly known as CtBP/BARS, is known to participate in the process of membrane trafficking and Golgi partitioning during mitosis (25, 26) as well as regulate membrane composition in cancer cells (40). In addition to CtBP1, the *CtBP2* gene also produces two splice variants of CtBP2 known as CtBP2-L and CtBP2-S, as well as a third isoform known as RIBEYE, which functions predominantly in the cytoplasm at ribbon synapses and has been extensively characterized in sensory neurons and bipolar cells (37, 41–43). Nonetheless, while the non-nuclear functions of CtBP isoforms are important, they will not be discussed in further detail here.

CtBPs and transcriptional activation

Recent studies suggest a novel function for CtBPs in transcriptional activation. For example, *Drosophila* CtBP (dCtBP) performs corepressor functions that are largely consistent with the function of mammalian CtBP homologues. However, a recent study suggested that dCtBP is able to modulate Wingless signaling through both repression and activation of the Wingless target genes (19, 44). Although the precise mechanism by which this activation occurs is not well characterized, evidence suggests that the role of dCtBP in determining if Wingless targets are activated or repressed is largely dependent on its dimerization state (19). Whereas dCtBP dimers exert transcriptional repression in this context, dCtBP monomers are capable of stimulating transcription of Wingless targets (19). dCtBP also plays a putative role in the control of *Drosophila* clock genes and regulation of circadian rhythms as overexpression of this protein increases mRNA expression of a number of clock genes *in vivo*. *In vitro* analysis of a potential mechanism for this regulation of clock genes revealed that dCtBP requires the presence of the transcriptional activator, CLOCK/CYCLE (45). This suggests that some transcriptional activation by dCtBPs may be mediated by the association of these proteins with classical transcriptional activators. Following the description of context specific transcriptional activation by CtBPs in *Drosophila*, similar activity was attributed to mammalian CtBPs in relation to Rac, a member of the Ras superfamily of small GTPases that are central regulators of cell migration and oncogenesis, and the Rac-specific guanine nucleotide exchange factor (GEF) Tiam1 (46, 47). Nonetheless, examples of transcriptional activation by CtBPs are rare and require further analysis before firm mechanistic conclusions on this activity can be made.

Regulation of CtBPs

Post-translational modifications

Although CtBPs are regulated by a number of factors, post-translational modifications are the best characterized (21, 22, 48–52). For example, phosphorylation of CtBPs often targets these proteins for ubiquitination and subsequent proteasomal degradation. In many cases, when degradation of CtBPs occurs under stress conditions, this leads to cell death. Among the stress-activated kinases that phosphorylate CtBPs, homeodomain-interacting protein kinase 2 (HIPK2) and c-Jun N-terminal kinase (JNK) are the best characterized (51, 52). Both of these proteins have been shown to phosphorylate CtBP1 at Ser422 in response to damaging ultraviolet (UV) irradiation of cancer cells, but only HIPK2 has been demonstrated to

also phosphorylate CtBP2 at the homologous site (Ser428) (51, 52). Phosphorylation at Ser422 results in enhanced ubiquitination of CtBP1 and proteasomal degradation, leading to derepression of CtBP target genes (Figure 3A). It has also been demonstrated that CtBP1 can be phosphorylated by AMP-activated protein kinase (AMPK) at Ser158 in response to metabolic stress such as glucose withdrawal (49). Similar to phosphorylation of Ser422 by JNK and HIPK2, phosphorylation at Ser158 by AMPK results in ubiquitination of CtBP1 and degradation, thus inhibiting CtBP repressor function. Meanwhile, p21-activated kinase 1 (PAK1) also phosphorylates CtBP1 at Ser158, but intriguingly, it does not trigger ubiquitination or degradation of this corepressor, although phosphorylation at this site appears to suppress the corepressor activity of CtBP1 directly (48). This occurs *via* a transient loss of nuclear CtBP localization, which occurs in conjunction with a conformational change in CtBP1 structure resulting in loss of both corepressor and dehydrogenase activity in the presence of NADH, following phosphorylation by PAK1. Thus, although both AMPK and PAK1 suppress CtBP function, the fate of CtBP may be determined in a context-specific manner, with cellular stress inducing CtBP degradation and subsequent apoptosis, whereas CtBP phosphoylation stimulates downstream of mitogenic factors, which promote transient suppression of CtBP function that may be required for pro-survival signaling to occur (53). Lastly, CtBP1 can also be phosphorylated and targeted for proteasomal degradation by Akt at Thr176, a reaction that is facilitated by interactions of Pc2 with both CtBP1 and Akt (50). This interaction causes these proteins to localize to polycomb groups, which are known to function in transcriptional repression. The effect of phosphorylation on CtBP expression and stability is summarized in Figure 3.

The polycomb protein, Pc2, also plays another role in the regulation of CtBP1 by acting as a SUMO E3 ligase for

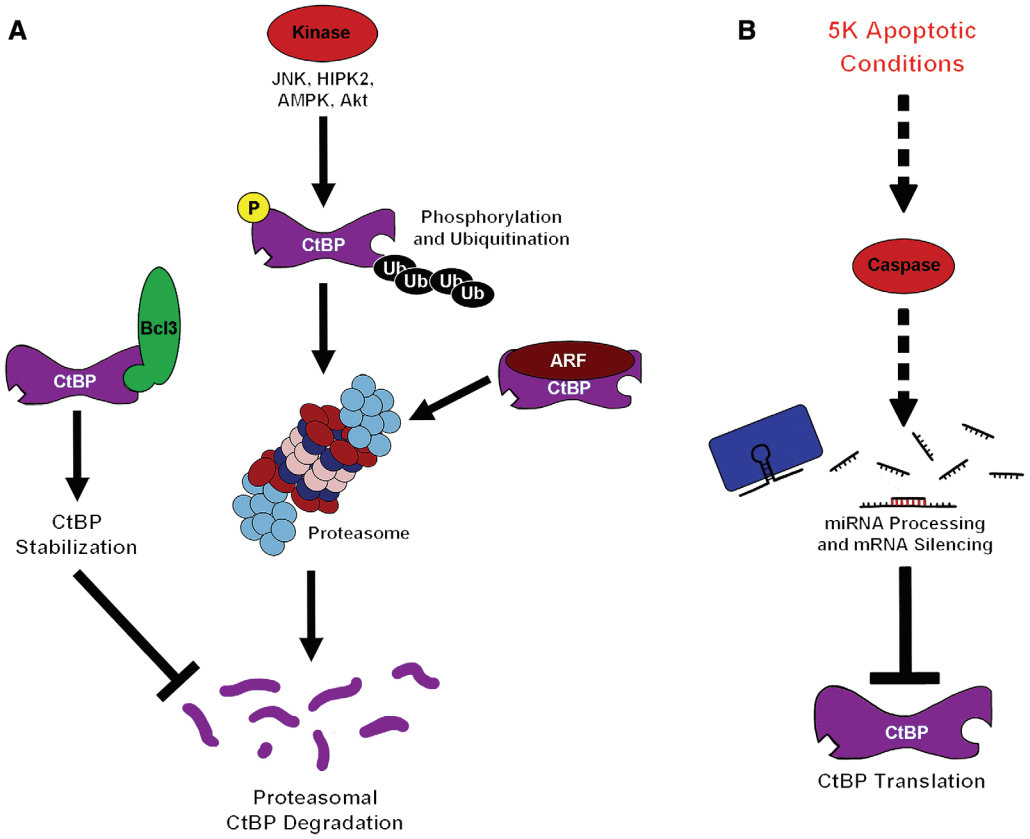

Figure 3 Regulation of CtBPs.
(A) Under conditions of cellular stress, CtBPs are phosphorylated by a number of kinases (e.g., JNK, Akt) that target CtBPs for ubiquitin-dependent proteasomal degradation. In addition to kinases, ARF interacts with CtBP2 through a hydrophobic region within the ARF protein, and this interaction leads to proteasomal degradation of CtBP2 in a phosphorylation- and ubiquitin-independent manner. Finally, CtBPs are stabilized by a PXDLS-dependent interaction with Bcl-3, a pro-survival member of the Bcl-2 family of proteins. (B) Under 5K apoptosis conditions, CtBPs are downregulated in neurons undergoing apoptosis in a mechanism that is dependent on intact miRNA biomachinery.

this protein (30). Although CtBP2 can also be SUMOylated by the same factors that SUMOylate CtBP1, this process occurs to a much lesser extent, and its effect on CtBP2 activity is not currently understood (30). SUMOylation has been shown to regulate a number of corepressors and their associated complexes, and SUMOylation of CtBP1 in particular, appears to regulate the localization of this protein within the cell (23, 54). CtBP1 is SUMOylated at Lys428, leading to retention of CtBP1 in the nucleus. This modification of CtBP1 directly opposes interactions with proteins such as nNOS, which enhance cytoplasmic localization of CtBP1 (23). Indeed, mutants lacking this residue in the SUMOylation consensus sequence have a largely cytoplasmic distribution and this correlates with decreased corepressor activity.

Several members of the SUMOylation machinery are known interacting partners of CtBP1. In particular, Ubc9 was identified as part of the core corepressor machinery that associates with and SUMOylates CtBP1 (10, 30). Interestingly, although Ubc9 is sufficient to SUMOylate CtBP1 *in vitro*, it has also been found that this reaction is facilitated by Pc2. Indeed, Pc2 interacts with both CtBP1 and Ubc9 as an E3 SUMO ligase to enhance the repressor activity of CtBP1 (30). Moreover, the interaction between CtBP1 and the SUMOylation machinery during transcriptional repression may act as a SUMOylation center for other members of the corepressor complex as a number of these factors are known to be regulated by SUMOylation (10, 54). In particular, HDAC1 is modified by SUMO, which increases its HDAC activity and enhances transcriptional repression (55). Moreover, SUMOylation of this protein regulates its association with different members of the corepressor complex, decreasing its interaction with CoREST, and increasing its association with ZNF198, another member of the CoREST complex that associates with CtBP (56). This illustrates that SUMOylation not only regulates the activity of various components of the corepressor complex, but it also may regulate their localization within the complex to decrease or enhance transcriptional repression. Other members of the corepressor complex that can be SUMOylated include LSD-1, CoREST, Znf198, and Pc2 (56). ZEB1, a repressor that is known to mediate its activity through the CtBP corepressor complex, is also SUMOylated. ZEB1 SUMOylation has been suggested to disrupt interactions with the corepressor complex (10); however, this has not been demonstrated experimentally, and previous evidence suggests that SUMOylation of ZEB1 is required for this protein to achieve its full function as a transcriptional repressor (57). Although the effect of SUMOylation is not clear for all members of the corepressor complex, it is notable that a number of proteins within the complex contain SUMO-interacting motifs (SIMs), which suggests that SUMOylation could play a role in the recruitment of complex members, and in maintaining the overall stability of the corepressor complex over time (54).

CtBP1 and CtBP2 are nearly identical in their domain structure and function; however, CtBP2 possesses an additional 20 amino acids at its N-terminus that play a significant role in regulating its function. In particular, CtBP2 possesses three lysine residues at positions 6, 8, and 10, all of which can be acetylated by the transcriptional activator p300 (22). Although acetylation does occur at all three residues, acetylation at Lys10 is particularly required for nuclear retention of CtBP2 (22). It is also noteworthy that CtBP activity can be regulated by acetylation of CtIPs such as E1A and RIP140. In both cases, acetylation of lysine residues flanking the PXDLS-binding motif located in these proteins results in decreased CtBP association and reduced transcriptional repression (58–60). Thus, CtBP activity can be regulated indirectly by enhancing or inhibiting its ability to bind CtBP-interacting partners.

Metabolic and redox sensitivity of CtBPs

Similar to 2D hydroxyacid dehydrogenases, CtBPs contain a Rossman fold that is necessary for NADH binding. Thus, there has been speculation that these proteins may alter their activity based on the metabolic and redox state of the cell. Although both forms of this dinucleotide are capable of inducing CtBP dimerization, CtBPs bind NADH with up to 100-fold higher affinity than NAD+, suggesting that these proteins are in fact sensitive to the ratio of NADH to NAD+ within the nucleus (18, 61, 62). Nonetheless, additional reports have suggested that CtBPs bind both NAD+ and NADH with similar affinity (17, 63). Therefore, further investigation is required to determine which binding paradigm is correct.

Although CtBPs function as dehydrogenases, NADH regulation is more likely involved in increasing the stability of CtBP dimers rather than stimulating any catalytic activity (64). This is most clearly demonstrated by mutant forms of CtBPs lacking either the capacity to bind NADH or dehydrogenase activity. Whereas the latter mutants are capable of mediating corepressor activity, CtBPs deficient in dinucleotide-binding capacity are expressed at a lower level, suggesting they are inherently less stable and are functionally impaired in terms of corepressor activity (64). This is contrary to a previous report demonstrating that CtBP2 mutants

lacking dehydogenase activity display corresponding decreases in corepressor activity (20). These opposing observations could be reconciled by findings in a recent study suggesting that some catalytic mutants of CtBP also display reduced binding affinity for NADH, although these mutants are still capable of forming CtBP dimers (63). Furthermore, a study by Madison et al. (63) recently proposed that CtBPs not only form functional dimers, but also higher-order tetramers that are also essential to CtBP function and are controlled by a tryptophan residue (Trp318) that acts as dimerization switch following binding to NADH.

Consistent with this idea, NADH binding has been previously shown to conformationally alter CtBP to adopt a 'closed' state that is more amenable to CtBP oligomerization (17). Moreover, increasing concentrations of NADH have been shown to increase interactions with other proteins containing a PXDLS-binding motif by inducing oligomerization, enhancing CtBP binding to transcriptional repressors, and increasing transcriptional repression both *in vitro* and *in vivo* (20, 62). Collectively, these results suggest that CtBPs are indeed sensitive to the NAD+/NADH ratio, and by extension, the metabolic and redox state of the cell. Given that the redox balance and metabolic state of the cell is significantly affected by a variety of cellular events, including growth, oncogenesis, and apoptosis, it is likely that the NAD+/NADH ratio plays an important role in determining the functions of CtBPs in these processes. However, a direct correlation between the metabolic and redox state of the cells and CtBP activity has not been definitively established in these processes and warrants further investigation.

Other regulators of CtBPs

Whereas post-translational modifications and NADH binding are currently the best characterized modes of regulating CtBP activity, other protein regulators are beginning to emerge. As a result, the role of CtBPs in a number of cellular processes is expanding, revealing these proteins to be the target of many signaling pathways involved in cell death and survival as well as onocogenesis. Indeed, a recent study revealed that caspases indirectly mediate the downregulation of CtBPs in neurons under some cellular conditions as removal of depolarizing potassium from cerebellar granule neuron (CGN) cultures (5K apoptotic conditions) was toxic and resulted in reduced expression of CtBP1 and CtBP2 in a caspase- and micro-RNA (miRNA)-dependent manner (Figure 3B) (65). In addition to caspases, CtBPs are also regulated by the pro-survival protein Bcl-3 (Figure 3A). Bcl-3 protein interacts with CtBP1 in a PXDLS-dependent manner to stabilize CtBP1 expression within the cell (66). This process is mediated by blocking CtBP1 proteasomal degradation and results in suppression of pro-apoptotic genes (66).

In addition, recent evidence also demonstrates that CtBPs can also be regulated by the tumor suppressor alternative reading frame (ARF), which interacts with CtBP2 through a hydrophobic region within the ARF protein located between residues 46 and 51, and this interaction leads to proteasomal degradation of CtBP2 and cell death (Figure 3A). The mechanism by which ARF represses CtBP expression is presently unknown as ARF does not appear to cause the phosphorylation of CtBP2 nor does it increase CtBP2 ubiquitination beyond basal levels, both of which target CtBP to the proteasome (67).

Finally, CtBPs may also be regulated by their localization to specific areas of the cell, or even to specific proteins by other CtBP-interacting partners. For instance, CtIP has been demonstrated to recruit CtBPs to proteins such as oncogenic proteins Rb and BRCA (68, 69), although CtIP does not always require CtBP recruitment to mediate transcriptional repression (70–72). Thus, several studies have demonstrated that CtBPs are regulated by many signaling pathways that are involved in cellular survival and death.

CtBPs in development

Invertebrates and non-mammalian models

Given the diverse and critical functions of CtBPs, it is perhaps not surprising that these proteins have important functions during development. Indeed, mRNA transcripts of CtBPs are widely expressed (6, 73–75). Further, zygotic mutations of dCtBP are embryonic lethal, suggesting an important involvement of dCtBP in development (76–78). The involvement of dCtBP during embryonic development is consistent with its role in transcriptional repression as CtBPs are recruited by short-range repressors such as Snail, Knirps, and Krüppel (79, 80). In addition to functioning during *Drosophila* development, CtBPs also regulate *Xenopus* embryonic development, as expression of an xCtBP fusion protein designed to enhance CtBP transcription resulted in loss of head, eyes, and shortened anterior-posterior axes (81). Although CtBPs have essential functions during development, relatively few studies have examined the precise involvement of CtBPs in central nervous system (CNS) development or neuronal survival.

CtBPs in avian and murine development

During avian development, although CtBP1 and CtBP2 are often expressed in the developing embryo in overlapping domains, CtBPs also exhibit region- and temporal-specific expression patterns. For instance, although only CtBP2 is expressed in the primitive streak during early development, both CtBP transcripts are expressed in this domain at later developmental stages (82). These findings are in agreement with murine studies that also demonstrate duplicative and unique roles for CtBP1 and CtBP2 during development and CNS maturation. For instance, although CtBP1 homozygous null mice are small and show a 23% reduction in viability at P20, those that do survive are fertile (Figure 4) (6). In contrast, CtBP2 homozygous null mice fail to develop beyond E10.5 and demonstrate axial truncations and delayed development of the forebrain and hindbrain (6). In support of overlapping functions for CtBP1 and CtBP2, the generation of various compound mutant mice resulted in dosage-sensitive developmental defects. For example, reducing the expression of CtBP2 in CtBP1$^{-/-}$ mice exacerbated the CtBP1$^{-/-}$ phenotype as CtBP1$^{-/-}$ CtBP2$^{+/-}$ mice died *in utero* and exhibited increased developmental defects and abnormalities in the formation of several skeletal structures, such as failure to generate cartilaginous and fully ossified skeletal elements in the ribs and vertebrae. In a similar manner, CtBP1$^{+/-}$ CtBP2$^{-/-}$ mice also demonstrated enhanced developmental defects such as failure to complete neural tube closure. This is a marked difference from CtBP1$^{+/+}$ CtBP2$^{-/-}$ mice, which completed neural tube closure. Finally, mice homozygous null for both CtBP1 and CtBP2 demonstrated the most severe embryonic phenotype displaying minimal heart morphogenesis, blebs and blisters in the neuroectoderm, and earlier embryonic death at E9.25 (6). These findings demonstrate that CtBP1 and CtBP2 exhibit both unique and redundant functions during mammalian development.

This study described above examining CtBPs during murine development is consistent with normal human tissues and human cancer cell lines, which also demonstrate overlapping and unique expression of CtBP1 and CtBP2 (75). Nonetheless, expression of CtBP2 differs between humans and mice, particularly in skeletal muscle where CtBP2 expression is greater in humans (73). Therefore, in future studies, it will be important to dissect the functional significance of unique and overlapping patterns of CtBP1 and CtBP2 expression during development and CNS maturation.

CtBPs and CNS development

To date, the involvement of CtBPs in the nervous system has mostly been limited to the role of these proteins in development. For example, dCtBP negatively regulates the formation of mechanosensory bristles as loss-of-function dCtBP mutants exhibit supernumerary mechanosensory bristles, whereas gain-of-function dCtBP mutants demonstrate a marked loss of bristles (78, 83). dCtBP may influence the appearance of ectopic bristles through extrasensory organ precursor cells, which are also increased in loss-of-function dCtBP mutants (78). In addition to regulating the formation of mechanosensory bristles, dCtBP also promotes eye and antennal specification through interactions with Eyeless, Distal antenna, Distal antenna related, and Daschund proteins (27). Moreover, CtBP1 and CtBP2 also differentially mediate nervous system development in the developing chick.

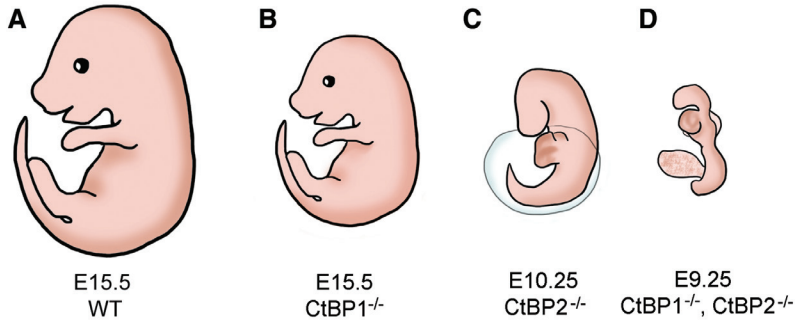

Figure 4 CtBPs in development.
(A) Wild-type mouse embryo. (B) CtBP1$^{-/-}$ knockout mouse. Note that CtBP1$^{-/-}$ knockout mice are small and viable but show a reduced survival rate. (C) CtBP2$^{-/-}$ knockout mouse. Note that CtBP2$^{-/-}$ knockout is embryonic lethal, and these mice show greater developmental defects when compared with CtBP1$^{-/-}$ knockout mice. (D) CtBP1$^{-/-}$/CtBP2$^{-/-}$ knockout mouse. Note that CtBP1/CtBP2-null mice demonstrate enhanced developmental defects and earlier embryonic death when compared with either CtBP1$^{-/-}$/CtBP2$^{+/+}$ or CtBP1$^{+/+}$/CtBP2$^{-/-}$ mice. E, embryonic day.

For instance, emerging neural crest cells express CtBP2, whereas dorsal root ganglia express CtBP1 (84). The importance of CtBP expression during avian CNS development is highlighted by the key involvement of these proteins in regulating the transition of neural precursor cells in the ventricular zone of the dorsal spinal cord from a proliferative to a differentiated state (85). Furthermore, as previously mentioned, CtBPs appear to have an important role in neural tube closure and development of the forebrain and hindbrain in mice (6). These previous reports highlight critical functions for CtBPs during development and specifically suggest an essential role for CtBPs in neuronal development.

Other functions of CtBPs in the nervous system

In addition to neuronal development, recent reports indicate that CtBPs may critically mediate other aspects of neuronal function in the adult murine brain. For instance, although the significance remains to be elucidated, nuclear expression of CtBP2 was higher in excitatory cells when compared with inhibitory cells of the hippocampus, whereas CtBP1 demonstrated higher nuclear expression in inhibitory cells (86). Moreover, although previous evidence suggests that CtBP1 functions principally in the nucleus, CtBP1 expression has also been demonstrated in pre-synaptic terminals of cultured hippocampal neurons, highlighting a potential function for CtBP1 in learning and memory that is distinct from its role as a transcriptional corepressor (38). In agreement with a pre-synaptic function for CtBPs, CtBP2 also colocalized with the pre-synaptic marker Bassoon in the cerebellum, and to a lesser extent, the hippocampus and cerebral cortex. The NLS is absent from the alternative splice variant CtBP2-S, which appears to be the major CtBP2 isoform localized to synapses. In contrast, CtBP2-L contains the NLS and localizes more exclusively to neuronal nuclei (86). The localization of CtBP1 and CtBP2 to pre-synaptic terminals may indicate a function for CtBPs in neurotransmitter release as CtBP1 has been implicated in both membrane fission and fusion (87). Furthermore, RIBEYE, is the main component and scaffold for ribbon synapses in sensory neurons such as photoreceptor and hair cells, suggesting an important function for RIBEYE in fine-tuning the tight release of synaptic vesicles required to detect a wide range of stimulus intensities important for proper vision and hearing (38). These previous reports suggest several important functions for CtBPs in the adult brain, which are both consistent with and distinct from their well described role in transcriptional repression.

CtBPs in apoptosis

Consistent with their major roles in transcriptional corepression, early studies demonstrated that CtBPs promote cellular survival primarily through repression of pro-apoptotic molecules of the Bcl-2 family of proteins (Figure 5). For instance, mouse embryonic fibroblasts (MEFs) deficient in both CtBP1 and CtBP2 expression were hypersensitized to apoptosis initiated by diverse stimuli such as Fas ligand, staurosporine, and UV irradiation. Furthermore, increased expression of the pro-apoptotic molecules PERP, Bax, Bik, Puma, p21, and Noxa was observed in CtBP-deficient MEFs (Figure 5) (7, 88, 89), and enhanced expression of both PERP and Bax was rescued by introduction of CtBP1 (Figure 5) (7, 88). Indeed, expression

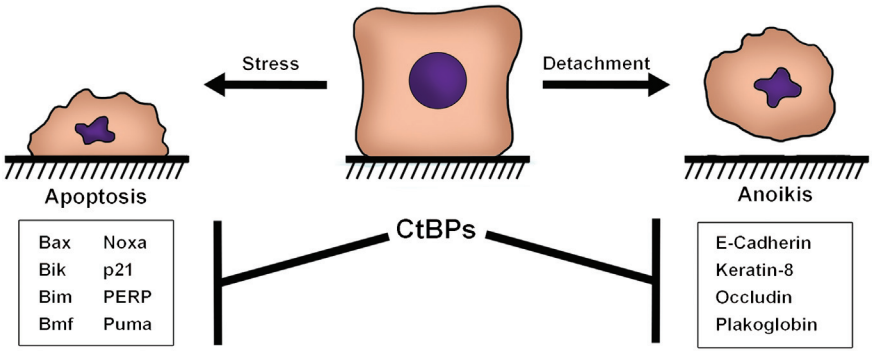

Figure 5 CtBPs as repressors of apoptosis and anoikis gene programming.
CtBPs promote cellular survival through transcriptional repression of the pro-apoptotic molecules Bax, Bik, Puma, Noxa, PERP, p21, Bim, and Bmf. CtBPs also repress epithelial gene programming associated with anoikis insensitivity by repression of E-cadherin, occludin, plakoglobin, and keratin 8.

of active caspase 3, the death executioner of apoptosis, and its cleaved substrate, poly(ADP-ribose) polymerase (PARP), were reported in CtBP-null MEFs exposed to UV radiation (90). Furthermore, osteosarcoma cells subjected to siRNA knockdown of CtBP2 demonstrated enhanced expression of the pro-apoptotic Bcl-3 homology 3 domain (BH3)-only proteins Bim and Bmf (89). It is important to note that transcriptional repression of pro-apoptotic genes does not necessarily require the dehydrogenase activity of CtBP, as mutants defective in dehydrogenase activity inhibited apoptosis to a similar degree as wild-type CtBP (7).

CtBPs and p53-independent pathways

Although most, if not all, of the aforementioned genes that are repressed by CtBPs are transcriptional targets of the pro-apoptotic molecule p53, loss of CtBP function appears to induce apoptosis through a p53-independent mechanism in many cell systems. For example, siRNA against CtBPs or genetic knockout of CtBPs resulted in caspase activation and subsequent apoptosis in p53-deficient H1299 cells (90). CtBPs also interact with a BTB domain-containing protein, CZBTB38, in p53 knockout MEFs to enhance caspase-3 activation and apoptosis (91). Collectively, these data demonstrate that degradation of CtBPs can induce apoptosis by a mechanism that is not dependent on the tumor suppressor p53.

In UV radiation-induced apoptosis, HIPK2 has emerged as a key regulator of CtBP downregulation. Although HIPK2 typically induces apoptosis in response to UV radiation by phosphorylating and activating p53, HIPK2 can also induce cell death in a p53-independent manner through phosphorylation of Ser422 and subsequent proteasomal degradation of CtBP1. These results are also consistent with previous reports that HIPK2 can signal cell death either through p53 or in a p53-independent manner through the activation of JNK. Intriguingly, either UV radiation or JNK activation resulted in phosphorylation of CtBP1 on Ser422, proteasomal degradation, and apoptosis of human lung cancer cells (51). The effector function of HIPK2 on JNK may be modulated through SUMOylation as human HIPK2 is SUMOylated *in vitro* and this modification inhibited HIPK2-dependent JNK activation and subsequent apoptosis of p53-deficient Hep3B hepatoma cells (92). Although downregulation of CtBPs induces apoptosis in cells lacking p53, experimental evidence also demonstrates that CtBP may regulate the expression of p53 under certain cellular conditions in cancer cells (93, 94).

CtBPs and neuronal survival

Although largely unexplored, the essential involvement of CtBPs in mediating survival in non-neuronal cells and their central roles in nervous system development highlights a potential function for CtBPs in regulating neuronal survival. Indeed, we recently reported that CtBPs undergo caspase-dependent downregulation in primary CGNs exposed to a variety of neurotoxic insults, including removal of depolarizing potassium (5K apoptotic conditions), incubation with the BH3-only mimetic HA14-1, the nitric oxide donor, sodium nitroprusside, or the complex I inhibitor, 1-methyl-4-phenylpyridinium. In addition, CtBPs also undergo downregulation in N27 dopaminergic cells exposed to 6-hydroxydopamine. Further establishing an essential pro-survival function for CtBPs in CGNs, either antisense mediated downregulation of CtBP1 or treatment with the CtBP inhibitor, 4-methylthio-2-oxobutryic acid (MTOB), induced significant neuronal apoptosis concomitant with an increase in the pro-apoptotic BH3-only protein Noxa (65). Intriguingly, although the degradation of CtBP1 occurred in a caspase-dependent manner in CGNs exposed to 5K apoptotic conditions, the kinetics of CtBP1 degradation were not enhanced and recombinant CtBP1 was not cleaved *in vitro* by caspase 3. Furthermore, 5K apoptotic conditions did not have a significant effect on CtBP transcript expression. However, mouse embryonic stem cells displayed caspase-dependent downregulation of CtBP1 following exposure to staurosporine, an effect that was not observed in DGCR8 knockout cells that are deficient in miRNA processing. Therefore, caspases appear to regulate the expression of CtBP indirectly at a post-transcriptional level *via* a mechanism that is dependent upon miRNA biomachinery (Figure 3B). These data demonstrate that CtBPs can undergo caspase-dependent downregulation in neurons undergoing apoptosis as an alternative mechanism to proteasomal degradation, as incubation with the proteasome inhibitor MG132 did not prevent the downregulation of CtBPs in CGNs exposed to 5K apoptotic conditions. This report identifies a previously undescribed role for CtBPs in maintaining the survival of primary neurons and further underscores that the downregulation of CtBPs can be triggered in neurons by exposure to a variety of neurotoxic insults that have previously been implicated in neurodegenerative disease.

CtBPs and Rac GTPase

Intriguingly, we have also reported a significant loss of CtBPs during CGN apoptosis induced by the small

GTPase inhibitors, *Clostridium difficile* Toxin B (ToxB), and *Clostridium sordellii* lethal toxin (LTox) (65). As ToxB (an inhibitor of Rac, Rho, and Cdc42) and LTox (an inhibitor of Rac, Ras, and Rap) have overlapping specificity for inhibiting Rac GTPase, these findings suggest that Rac GTPase may function upstream of CtBPs to regulate their expression and consequently, the transcriptional repression of BH3-only pro-apoptotic proteins, such as Noxa (Figure 3). Although the precise mechanism by which CtBPs are downregulated in neurons exposed to small GTPase inhibitors requires further investigation, recent studies have suggested a positive correlation between the expression of CtBPs and the activity of Rho family GTPases in non-neuronal cells. For example, overexpression of CtBP2 paradoxically enhances the transcription of the Rac GEF Tiam1 and subsequently enhances the Rac-dependent migration of human non-small cell lung carcinoma cells (46). In conjunction with our own findings, these data suggest that CtBP1/CtBP2 and Rac GTPase may function in a positive feedback loop to regulate the expression and activities of one another. Nonetheless, it is important to consider that our studies demonstrating that CtBPs are regulated downstream of Rac GTPase were conducted in primary neurons, while the CtBP2-dependent upregulation of Tiam1 and subsequent activation of Rac GTPase was shown in H1299 cell lung and HCT116 colon carcinoma cells. Thus, whether or not Rac GTPase lies upstream or downstream of CtBP1 and CtBP2 may simply depend on the cell type. In future studies, it will be important to determine whether CtBP2 similarly triggers Tiam1-dependent activation of Rac GTPase in neurons and whether this has an effect on neuronal survival. Indeed, the link between CtBPs and Rho family GTPases is only recently being elucidated and future studies will be necessary to decipher the precise relationship between CtBPs and Rho family GTPases in neuronal survival.

CtBPs in Huntington disease and traumatic nerve injury

Although not directly examined in human neurodegenerative disorders, previous *in vitro* and *in vivo* animal studies have linked dysregulated CtBP activity to the progression of various neurodegenerative diseases, such as Huntington disease (HD). HD is an autosomal dominant neurodegenerative disorder caused by inheritance of one mutant huntingtin (Htt) gene. Mutant Htt (mHtt) contains an expanded polyglutamine repeat near the N-terminus and cleavage of mHtt results in an N-terminal fragment that is toxic to medium spiny striatal neurons when it is localized to the nucleus. Intriguingly, Htt contains a canonical PXLDS CtBP-binding site, suggesting that Htt may be involved in transcriptional repression. In human fibroblasts, full-length wild-type Htt interacts with CtBP in the nucleus to cause constitutive repression, and while a polyglutamine expansion in Htt impaired this interaction, mHtt that was targeted to DNA remained effective at transcriptional repression (95). Thus, mHtt may have a weaker interaction with CtBPs when compared with wild-type Htt, leading to instability of protein complexes required for CtBP-dependent transcriptional repression of pro-apoptotic genes.

In addition to the potential involvement of dysregulated CtBP function as a factor underlying the progression of HD, recent studies have also begun to elucidate the involvement of CtBPs in regulating neuronal degeneration following neuronal trauma. Following traumatic brain injury, both HIPK2 and CtBP2 were increased in the peritrauma brain cortex when compared with contralateral cerebral cortex. However, while HIPK2 was associated with activation of caspases and neuronal apoptosis, enhanced CtBP2 expression was associated with activation and proliferation of astrocytes (96). In contrast, CtBP2 was downregulated in Schwann cells following sciatic nerve crush in rats (97). Thus, these studies indicate that CtBP2 may play a role in regeneration following peripheral nerve injury. Nonetheless, the involvement of CtBPs in neuronal trauma are only beginning to be understood and further research is necessary to determine the precise involvement of CtBPs in traumatic nerve injury and regeneration.

CtBPs and neuroinflammation

Intriguingly, CtBPs may also have important functions in maintaining neuronal survival through their role in regulating the inflammatory response by microglia and astrocytes. In a recent study by Saijo et al. (98), it was demonstrated that the endogenous estrogen receptor β ligand, 5-androsten-3β,17β-diol (ADIOL), mediates the recruitment of CtBP1 and CtBP2 to the promoter region of c-Jun/c-Fos AP1-dependent promoters, leading to the transcriptional repression of genes that are implicated in activation of the inflammatory response in Th17 T cells. The recruitment of CtBPs to DNA was dependent upon ERβ, but not 17β-estradiol, as synthetic ERβ-specific ligands promoted CtBP recruitment and prevented inflammation. Indeed, the ERβ-specific ligands, indazole-Br and indazole-Cl, induced the interaction between ERβ and CtBPs and inhibited the inflammatory response in microglia and astrocytes.

In contrast to this prior study suggesting that CtBPs are important molecules that exert transcriptional repression of pro-inflammatory genes, a recent study indicates that CtBPs may also function to induce the inflammatory response *in vivo* in a rat model of neuroinflammation. Indeed, CtBP2 expression was induced in activated microglia and astrocytes following lipopolysaccaride (LPS) administration to rats. Enhanced CtBP2 expression may contribute to LPS-induced inflammation as CtBP2 knockdown in cultured microglia prevented the release of tumor necrosis factor α (99). Thus, the precise involvement of CtBPs in regulating the inflammatory response likely varies depending on the expression of various toll-like receptors as well as ERβ receptors on microglia cells and astrocytes. Nonetheless, these findings are particularly interesting given the critical involvement of aberrant activation of microglia and astrocytes in the CNS during the progression of multiple neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Parkinson disease (PD). Therefore, future studies should be aimed at clarifying the involvement of CtBPs in regulating the inflammatory response of microglia and astrocytes. Given their pro-survival function in primary neurons and the potential to repress inflammation, CtBPs may represent a novel therapeutic target for the treatment of various neurodegenerative diseases.

CtBPs in anoikis

The term 'anoikis' refers to a specific type of apoptotic death that occurs in response to loss of cell adhesion (100). This process is mediated by integrins that transmit intracellular signals in response to mechanical forces due to contact with the extracellular matrix (ECM). It is important for an organism to be able to remove displaced cells to prevent their reattachment to new matrices. Despite a unique definition, anoikis is an apoptotic process that can occur *via* either extrinsic or intrinsic apoptotic cascades (101, 102). Anoikis that is executed through the intrinsic apoptotic cascade is largely carried out by the pro-apoptotic BH3-only molecule Bim, and to a lesser extent, Bid (103). Following detachment from the ECM, extrinsic apoptosis can also be activated *via* increased expression of Fas and Fas ligand (104). Both the intrinsic and the extrinsic anoikis pathways converge on activation of the death executioner of apoptosis, caspase 3.

Evidence suggests that CtBPs are important modulators of anoikis as enhanced activation of CtBPs represses the expression of epithelial genes in parallel with the acquisition of anoikis insensitivity (Figure 5) (4). As epithelial cells contain prominent cell-cell and cell-matrix adhesions, repression of epithelial genes such as E-cadherin is an important process in acquiring anoikis insensitivity, allowing cells to survive following detachment from the ECM (100). MEF cells derived from CtBP1/2 double knockout mice displayed enhanced expression of several epithelial-specific proteins, such as E-cadherin, plakoglobin, occludin, and keratin 8 (Figure 5) (7). In a CtBP1 rescue experiment, these effects were abrogated, underscoring an essential function for CtBPs in suppressing epithelial genes. The ability of CtBP to repress epithelial genes did not require dehydrogenase activity as a glycine 183 to alanine mutation did not attenuate the ability of CtBP1 to downregulate epithelial gene expression (4). However, it has since been proposed that this particular mutation may only partially disrupt the NADH-binding motif (105). In accordance with a function of CtBPs in conferring anoikis insensitivity, MEFs deficient in the expression of both CtBP1 and CtBP2 displayed enhanced sensitivity to detachment-induced anoikis as evidenced by increased nuclear fragmentation and caspase activation when compared with attached cells, both effects of which were attenuated by reintroduction of CtBP1 (4). Collectively, these data highlight CtBPs as regulators of anoikis and suggest that dysregulation of CtBPs may underlie the metastatic nature of anoikis-insensitive tumor cells.

CtBPs and cancer

The original identification of CtBPs as binding partners for E1A adenovirus suggested the involvement of CtBPs in regulating oncogenesis. Indeed, an early study identified that E1A mutants defective in their PXDLS domain enhanced transformation in conjunction with the Ras oncogene in rodent epithelial cells, suggesting that E1A may promote oncogenesis *via* inhibition of CtBPs. More recently, a genome-wide analysis was performed to provide a more comprehensive description of CtBP repression targets. From this study, CtBP targets could be categorized into three main categories: genes that regulate cell renewal and pluripotency, genes that regulate genome stability, and genes that regulate epithelial differentiation and prevent epithelial-to-mesenchymal transition (EMT) (40, 106). Remarkably, these three classes also describe pathways that are classically dysregulated in cancer. Indeed, many advances have been made in elucidating the role of CtBP in the pathogenesis of various cancers.

CtBP regulation of tumor suppressor genes

Phosphatase and tensin homologue (PTEN) is a known tumor suppressor that is involved in cell cycle regulation. It was discovered that overexpression of CtBP2 causes a decrease in PTEN expression levels and increases cell migration through activation of a PI3K-dependent pathway (46). Furthermore, the Snail transcription factor, which drives breast cancer metastasis, represses PTEN in a CtBP-dependent manner, which results in a pro-survival effect (79, 107).

In addition to PTEN, other tumor suppressors, such as p53 and hypermethylated in cancer (HIC1), are modulated by actions of CtBPs. Transcription of p53 target genes is negatively regulated through a direct physical interaction with the oncoprotein human double minute 2/mouse double minute 2 (Hdm2/Mdm2). Hdm2/Mdm2 is a known inhibitor of p53 that can inactivate this protein *via* multiple mechanisms, including proteasomal degradation, nuclear export, and reduced interaction with transcriptional coactivators. However, Hdm2 has also been demonstrated to recruit CtBP2 in a redox-sensitive manner to the promoter region of p53 to exert transcriptional repression. This interaction, which occurs through an acidic domain in Hdm2, is diminished under hypoxic conditions that increase the NADH/NAD+ ratio in MCF-7 breast cancer cells, resulting in the derepression of p53 (93). These findings are in contrast to the established paradigm that increased intracellular NADH levels promote CtBP interactions with proteins containing a PXDLS-binding motif, and indicate that the regulation of Hdm2/CtBP-mediated repression of p53 occurs *via* a distinct mechanism (20, 62), consistent with a role in transcriptional repression, loss of CtBPs led to enhanced expression of p53; however, p53 exerted a pro-survival effect in cells deficient in CtBPs *via* p53-dependent expression of $p21^{WAF1}$, which has previously been demonstrated to antagonize p53-dependent cell death (94, 108). Thus, whether or not p53 is required for cell death provoked by the downregulation of CtBPs in cancer cells likely occurs in a cell type- and context-specific manner. In a similar manner to Hdm2, the transcriptional repressing qualities of the tumor suppressor protein HIC1 are dependent on its interaction with CtBP1 (11). Thus, CtBPs function as critical modulators of various tumor suppressor proteins, whose regulation is consistently implicated in the etiologies of multiple cancers (Figure 6). Nonetheless, future studies will be critical to further define the downstream effects of CtBP repression of tumor suppressors and their putative causal involvement in the development of cancer.

Another important tumor suppressor that interacts with CtBP is the adenomatous polyposis coli (APC)

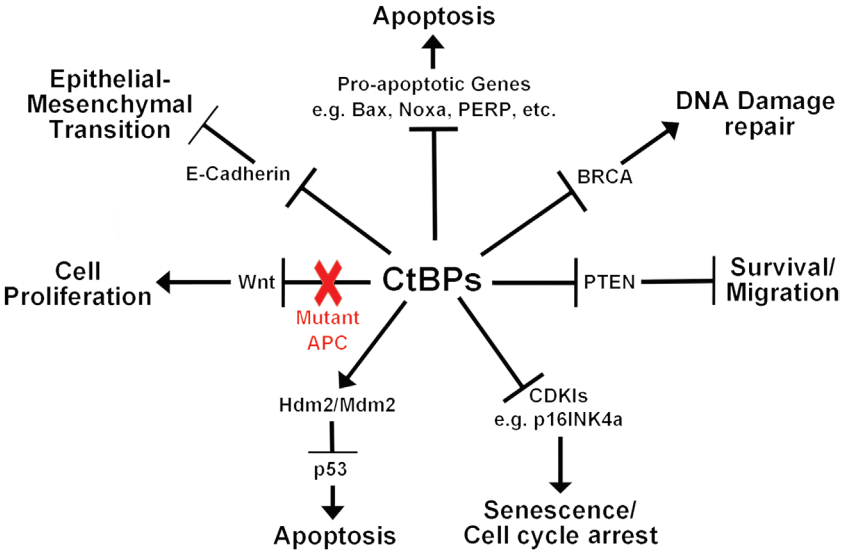

Figure 6 Dysregulation of CtBPs as a common pathological link in neurodegenerative disease and cancer.
CtBPs function to promote cellular survival through repression of pro-apoptotic (e.g., Bax, p53) and pro-anoikis (e.g., E-cadherin) gene programming. Typically, CtBPs suppress Wnt-dependent cell proliferation except in the case of mutant APC. CtBPs diminish DNA damage repair and cell cycle arrest. Finally, CtBPs enhance cell survival and migration through repression of PTEN. Thus, diminished expression of CtBPs may underlie aberrant neuronal apoptosis neurodegenerative disease, whereas enhanced CtBP expression may promote carcinogenesis by inhibition of apoptosis and stimulation of proliferation, migration, survival, and genomic instability.

protein, which typically interacts with CtBP to repress Wnt target gene expression. APC prevents transcription by targeting β-catenin for proteasomal destruction, promoting nuclear export, or through sequestration. Importantly, APC-mediated β-catenin sequestration relies on APC interacting with CtBP, suggesting that these proteins may exist in a complex to mediate repression of Wnt-dependent gene transcription (109). Indeed, this association has been demonstrated to be important in repression of the c-myc oncoprotein (110). Mutations in *APC* disrupt the interaction between APC and CtBP, a process that is a causal factor in aberrant Wnt signaling in cancer. Specifically, APC loss-of-function mutations are observed in most cases of colorectal cancer, including the familial adenomatous polyposis (FAP) syndrome. Furthermore, these interactions are also impaired in colorectal cancer cells harboring mutations in *APC*, suggesting a causal relationship between APC-CtBP complex function and Wnt pathway signaling in cancer progression (Figure 6) (110).

CtBP also regulates the expression of several cyclin-dependent kinase inhibitors (CDKIs), known for their onco-suppressive properties through modulating cell cycle arrest. Specifically, increased CtBP expression negatively regulates the tumor suppressor p16INK4a and prevents cell senescence in human esophageal squamous cell carcinoma, suggesting that CtBP levels may have a direct role in the evasion of cell cycle regulation in this tumor type (111, 112). Moreover, CtBP has been shown to repress expression of p21 (waf1/cip1) in a PARP-dependent manner during DNA damage conditions known to be prevalent in tumor micro-environments (113). Although CtBP modulation of the cell cycle remains to be demonstrated in many tumor types, the implications of CtBP-dependent repression of CDKIs highlight a key role for CtBPs in a crucial upstream event in the evasion of cell cycle arrest and tumor progression. The role of CtBPs in promoting cellular transformation through regulation of tumor suppressor genes is summarized in Figure 6.

CtBP repression by tumor suppressors

Although the role of CtBPs in repression of tumor suppressor genes is quite clear, *in vitro* studies also reveal that CtBP can be reciprocally repressed by the actions of other tumor suppressor proteins. As previously discussed, HIPK2 has been shown to mediate proteasomal degradation of CtBP through phosphorylation (51, 90), and reduced CtBP levels are associated with increased apoptosis, consistent with the tumor suppressive functions of HIPK2 (90). However, future studies will be required to discern the importance of this interaction in known cancers.

Mutations of ARF tumor suppressor are frequently associated with hepatocellular carcinoma (HCC) (114). Although the causal mechanistic effects of these mutations remain to be determined, the implications for its role in the development of cancer have yielded studies indicating a novel function of ARF in regulating CtBPs. *In vitro* studies in lung carcinoma cells have revealed that exogenous expression of the tumor suppressor ARF has an antagonistic effect on CtBP2-mediated cell migration through directly targeting CtBP for proteasomal degradation (46, 67). Similarly, decreased ARF levels correlate with increased CtBP levels in human colon adenocarcinoma, further supporting that ARF may function as a repressor of CtBP (115). In contrast, ARF repression of tumor migration requires the interaction between ARF and CtBP in HCC cell lines as well as pancreatic ductal adenocarcinoma models (116, 117). These data are interesting in the context of other studies that indicate repression of ARF may also occur *via* a CtBP-dependent mechanism, demonstrating that a complex feedback mechanism may be involved in CtBP/tumor suppressor regulatory pathways (111).

CtBP inhibition has also been demonstrated to occur through the activity of the familial colon cancer-linked tumor suppressor APC, possibly through proteasomal degradation of CtBP (109, 118, 119). APC is shown to target CtBP for destruction, which is important in ameliorating CtBP suppression of intestinal differentiation through retinol dehydrogenases (118). Accordingly, increased CtBP1 levels are observed in tumors obtained from FAP patients harboring mutations in APC, further implicating APC in the repression of CtBP expression (118, 119). These data are interesting in light of the previously described involvement of APC and CtBP in regulating Wnt signaling. The disparity may be due, in part, to cell type specificities in function. However, given the complex nature of CtBP regulation, it is also likely that a feedback mechanism between CtBP and APC functions to regulate these two critical proteins. Collectively, these data also suggest that CtBP represents a convergence point in a complex mechanism involving APC/β-catenin, Wnt, and other factors such as retinol dehydrogenase, and that dysregulation of this pathway due to mutations likely plays a significant role in the development of colon adenocarcinomas.

Breast cancer/BRCA regulation

Breast cancer 1 and 2 (BRCA 1, 2) proteins are potent tumor suppressors that function in DNA repair. Mutations

in these genes are consistently linked to several different types of cancer, most commonly, breast cancer. As is the case with other tumor suppressor proteins, *BRCA* regulation involves interactions with CtBP. Studies in head and neck squamous cell carcinomas reveal that *BRCA1* is repressed through CtBP1 binding to its promoter. Additionally, this interaction occurs in a redox-dependent manner and increases in hypoxic conditions associated with tumor micro-environments (120). Additional studies demonstrate that CtBP2 represses *BRCA1* in ovarian cancer cell lines (121) and that loss of CtBP at the *BRCA1* promoter results in increased *BRCA1* expression (122). Finally, CtBP1 is shown to repress *BRCA2* expression through interaction with the slug repressor protein in human breast cancer cells (123). Interestingly, CtBP1 exists in a complex with HDAC1 and p53 to inhibit *BRCA2* transcription (124). In contrast, studies in breast cancer-derived cell lines demonstrate that CtBP exerts a repressive function on in p53-mediated transcription (94). Collectively, these data describe a direct role for CtBP in the repression of *BRCA* and the development of breast cancer, which has proven to be a valuable mechanism in which to intervene therapeutically.

In contrast to data suggesting that CtBPs mediate breast cancer progression *via* repression of *BRCA* genes, it has also been demonstrated that CtBP is negatively regulated in breast cancer. MUC1 is an oncoprotein that is overexpressed in some forms of breast cancer. Importantly, this protein inhibits CtBP repression of cyclin D1 through interaction with TCF7L2, thus promoting cell cycle progression (125). Thus, the role of CtBPs in breast cancer, like other types of cancer, is complex and is likely regulated in cell type- and tumor micro-environment-specific contexts.

CtBP role in EMT

Loss of cellular adhesion and acquisition of anoikis resistance represents a critical step in the cellular transition to malignancy. This process involves a downregulation of epithelial-specific genes accompanied by an increase in expression of mesenchymal-specific genes, allowing for a more motile and invasive mesenchymal phenotype and is termed the 'epithelial-to-mesenchymal transition' (126). EMT allows for a tumor cell's motility and is mediated through downregulation of cellular adhesion molecules such as E-cadherin (127) as well as evasion of anoikis. Early studies demonstrated that several epithelial-specific genes are negatively regulated by CtBP, including E-cadherin, and plakoglobin (7). Importantly, CtBP recruitment to the E-cadherin promoter has been demonstrated to occur during hypoxic conditions that are present in metabolically active tumor types (128). Moreover, CtBP overexpression has been correlated with decreased E-cadherin levels in human colon cancers (129). Although CtBP is quite clearly involved in the repression of E-cadherin, and therefore involved in EMT, modulation of CtBP recruitment to the E-cadherin promoter is quite complex. Expression of E-cadherin is partially controlled through recruitment of CtBP by ZEB1 and ZEB2 (7, 24, 130). Specifically, overexpression of ZEB1 causes a decrease in E-cadherin in several cancers, including, breast cancer, uterine cancer, and colon cancer (131–135). Interestingly, ZEB1 has also been demonstrated to dissociate from CtBP to form an activation complex with SMADs in response to tumor growth factor (TGF) β signaling, which is associated with enhanced expression of mesenchymal-specific genes (130, 136). Collectively, these data suggest that an increase in ZEB1 levels likely causes an increase in CtBP recruitment and resultant repression of E-cadherin in various cancer types. Moreover, ZEB1 also enhances the expression of mesenchymal-specific genes in a CtBP-independent manner downstream of TGF-β signaling.

The regulation of cell adhesion proteins by CtBP is also a complex process. The cell adhesion-related phoshoprotein, pinin/DRS (Pnn), has been shown to directly interact with CtBP1 and interfere with its ability to repress E-cadherin expression (137). CtBPs are also regulated at the post-transcriptional level by the miRNA degradation machinery. Specifically, miR-137 is shown to have tumor suppressive properties in melanoma cells and increases E-cadherin levels through CtBP1 repression (138).

Other regulators of CtBP in cancer

The involvement of CtBP corepressor activity in colon cancers and breast cancers is perhaps the most extensively studied. However, CtBP transcriptional regulation has been demonstrated in a variety of other cancers. Upregulation of the ecotropic virus integration site 1 protein (Evi-1) is a hallmark of myeloid malignancies and myelodysplasia. Importantly, Evi-1 inhibition of TGF-β pathway through repression of SMAD involves recruitment of CtBPs (139). Incidentally, SMAD repression may also occur through recruitment of CtBPs by ZEB (130). Increased expression and mislocalization of CtBP is evident in prostate cancer cells. Furthermore, knockdown of CtBP in prostate cancer cell lines inhibited cell invasion (140). In melanoma, CtBPs are shown to repress BRCA1

and p16INK4a (141). Thus, dysregulation of CtBPs may be a common factor underlying the development of many different types of cancers.

Outlook

Targeting CtBPs in neurodegenerative disease

Recent evidence demonstrating that downregulation or inhibition of CtBPs is sufficient to trigger neuronal apoptosis underscores the potential therapeutic benefit of increasing the expression or activation of CtBPs as a therapeutic treatment option for neurodegenerative diseases (Figure 7A) (65). Indeed, this notion is supported by evidence suggesting that mHtt may have a weaker interaction with CtBPs when compared with wild-type Htt, leading to instability of protein complexes required for CtBP-dependent transcriptional repression and neuronal survival (95). In future studies, it will be of critical importance to determine whether loss of CtBP function underlies the neuronal cell death associated with diseases such as ALS, PD, and Alzheimer disease. In the context of HD and other neurodegenerative diseases for which loss of CtBP function may play a pivotal role in neuronal apoptosis in the CNS, it would be a worthy approach to perform a small molecule screen to identify compounds that function to stabilize the dimerization of CtBPs, thereby increasing their stability and activity. Furthermore, given the involvement of Rac GTPase in modulating the expression of CtBP in primary neurons, small molecule activators of a Rac-specific GEF (e.g., Tiam1) may also promote increased CtBP expression to enhance neuronal survival.

In addition, the potential involvement of CtBPs in suppressing inflammation through binding and repressing the transcription of genes that are implicated in activation of the inflammatory response suggests that targeting CtBP activation in microglia and astrocytes may also offer promising results for the treatment of neurodegenerative disease. In the clinic, the ERβ-specific ligand, indazole-Cl, has already been demonstrated to reduce inflammation in autoimmune encephalomyelitis (98), and therefore, ERβ-specific ligands represent potential therapeutic compounds for the treatment of neurodegenerative diseases in which inflammation plays a pathogenic role. In conclusion, although relatively few studies have examined the function of CtBPs in maintaining neuronal survival to date, recent studies have revealed that loss of CtBP function may contribute to neuronal cell death and ultimately neurodegenerative disease. Thus, enhancing CtBP function in both neurons and glial cells of the CNS offers a novel and largely unexplored therapeutic approach for the treatment of neurodegenerative diseases.

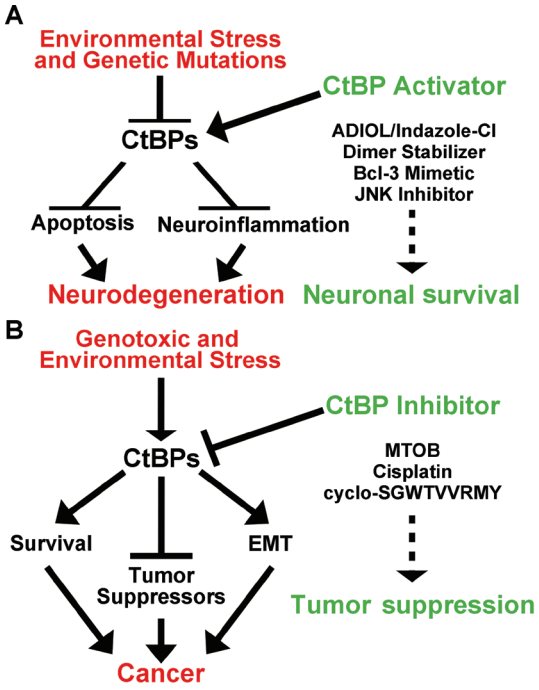

Figure 7 CtBPs as novel therapeutic targets for the treatment of neurodegenerative disease and cancer.
(A) Environmental stress and genetic mutations may induce neuronal apoptosis through diminished CtBP activity, ultimately resulting in neuroinflammation and neuronal cell death. Thus, restoration of CtBP activity *via* ADIOL, a compound designed to stabilize CtBP dimers, a Bcl-3 mimetic, or a JNK inhibitor may offer novel approaches to treat neurodegenerative diseases. (B) Genomic and environmental stressors may also enhance CtBP activity, resulting in increased cellular survival, EMT, and ultimately carcinogenesis. Utilizing CtBP inhibitors such as MTOB, cisplatin, or cyclo-SGWTVVRMY may lead to reduced CtBP activity and tumor suppression.

CtBPs as targets for cancer treatment

Increasing evidence supports a role for CtBPs in the conversion of healthy cells to a neoplastic phenotype. This occurs through regulation of several different pathways including the effect of CtBP on the expression of epithelial specific genes, as well as repression of tumor suppressor genes. Therefore, therapeutic inhibition of CtBP action may be a viable option for cancer treatments (Figure 7B).

MTOB is known to be cytotoxic to cancer cells and is a strong substrate for CtBP, thereby inhibiting the endogenous actions of CtBP. Examination of cell death induced by CtBP inhibition with MTOB indicated that this treatment promotes cell death in various cancer cell lines through displacement of CtBP from the pro-apoptotic Bik promoter (115). MTOB has also been shown to be effective in suppressing ovarian cancer cell-line survival in a CtBP-dependent manner (121). Importantly, these findings seem to be specific to malignant cells, which is a crucial factor in the physiological elimination of cancer cells (115).

Cisplatin is an alkylating agent commonly used as a chemotherapeutic agent in the treatment of cancer. Evaluation of human lung cancer cell lines demonstrated that cisplatin decreases CtBP levels in a JNK-dependent manner (51). This interaction has proven to be important in the development of chemotherapeutic agents, such as cisplatin, which may partially exert its effects through HIPK2 activation (142). In conclusion, the importance of CtBP involvement in cancer is highlighted by the development of therapeutic interventions that directly target CtBP actions. Furthermore, as CtBP is specifically dysregulated in tumor cell populations, and not in healthy cell populations, therapeutic targeting of CtBP dysregulation represents a unique approach to developing cancer cell-specific treatments. As studies continue into the mechanistic roles of CtBP in cancer development, new insight will be given into additional ways to modulate aberrant CtBP function in tumor cells.

Expert opinion

CtBPs play a critical role in development, cellular survival, and tumorigenesis. Although early studies highlighted an essential function for CtBPs in transcriptional corepression, more recent data have highlighted the involvement of CtBPs in additional cellular functions such as transcriptional activation and Golgi maintenance. At the cellular and organismal level, CtBPs have been well described as pro-survival molecules that are capable of repressing apoptosis, and more specifically, anoikis. Nonetheless, the involvement of CtBPs in promoting cancer and the potential loss of CtBP function in neurodegenerative disease are relatively unexplored concepts. Thus, future studies should aim to elucidate the precise involvement of CtBP dysregulation in *in vitro* and *in vivo* models of disease. In addition, inhibition or activation of CtBPs in cancer and neurodegenerative disease models, respectively, should be examined as a potential therapeutic approach for treatment of these devastating diseases. Future pre-clinical studies will likely highlight CtBPs and their interacting partners as novel therapeutic targets for the treatment of cancer and/or neurodegenerative disease.

Highlights

- CtBPs are well-described transcriptional corepressors.
- CtBPs repress many pro-apoptotic genes.
- Loss of CtBPs enhances sensitivity to apoptosis and anoikis in non-neuronal cells.
- Downregulation of CtBPs is sufficient to provoke neuronal apoptosis.
- A reduction in CtBP function may contribute to neurodegenerative disease.
- Enhanced expression of CtBPs can lead to EMT and may underlie the metastatic nature of anoikis-insensitive tumor cells.
- Enhanced CtBP function may contribute to the development of certain cancers.

List of abbreviations

ADIOL	5-androsten-3β,17β-diol
ALS	amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
APC	adenomatous polyposis coli
ARF	alternative reading frame
BKLF8	basic Krüppel-like factor 8
BRCA	breast cancer
CGNs	cerebellar granule neurons
CDIs	cyclin-dependent kinase inhibitors
CtBP	C-terminal binding protein
dCtBP	*Drosophila* CtBP
CtIPs	CtBP-interacting proteins
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
Evi-1	ecotropic virus integration site-1
FAP	familial adenomatous polyposis
GEF	guanine nucleotide exchange factor
HIC1	hypermethylated in cancer
HCC	hepatocellular carcinoma
HD	Huntington disease
HDAC	histone deacetylase
Hdm2/Mdm2	oncoprotein human double minute 2/mouse double minute 2
HIPK	homeodomain interacting protein kinase 2
HMT	histone methyltransferase
Htt	huntingtin
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LSD1	lysine-specific demethylase 1

LTox	Lethal toxin
MEFs	murine embryonic fibroblasts
NADH	nicotinamide adenine dinucleotide
NLS	nuclear localization signal
nNOS	neuronal nitric oxide synthase
PAK1	p21-activated kinase 1
PARP	poly(ADP-ribose) polymerase
PD	Parkinson disease
Pnn	phoshoprotein pinin
PTEN	phosphatase and tension homologue
SIM	SUMO-interacting motif
ToxB	Toxin B
ZEB	zinc finger E-box protein.

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