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Developmental Cell, Vol. 7, 677–685, November, 2004, Copyright ©2004 by Cell Press

# The APC Tumor Suppressor Binds to C-Terminal Binding Protein to Divert Nuclear β-Catenin from TCF

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## Summary

**Adenomatous polyposis coli (APC) is an important tumor suppressor in the colon. APC antagonizes the transcriptional activity of the Wnt effector β-catenin by promoting its nuclear export and its proteasomal destruction in the cytoplasm. Here, we show that a third function of APC in antagonizing β-catenin involves C-terminal binding protein (CtBP). APC is associated with CtBP in vivo and binds to CtBP in vitro through its conserved 15 amino acid repeats. Failure of this association results in elevated levels of β-catenin/TCF complexes and of TCF-mediated transcription. Notably, CtBP is neither associated with TCF in vivo nor does mutation of the CtBP binding motifs in TCF-4 alter its transcriptional activity. This questions the idea that CtBP is a direct corepressor of TCF. Our evidence indicates that APC is an adaptor between β-catenin and CtBP and that CtBP lowers the availability of free nuclear β-catenin for binding to TCF by sequestering APC/β-catenin complexes.**

## Introduction

Most colorectal tumors have inactivating mutations in *APC*, and APC loss is an early if not initiating event in colorectal tumorigenesis (Kinzler and Vogelstein, 1996). Almost all mutations produce APC truncations, and the majority of these terminate within or upstream of the mutation cluster region (MCR) in the middle of the coding region (Nagase and Nakamura, 1993). The main function of APC is to downregulate the transcriptional activity of β-catenin, a Wnt effector that binds to DNA binding proteins of the T cell factor (TCF) family to stimulate the transcription of Wnt target genes (Polakis, 2000). This function of APC appears to be critical in the suppression of colorectal tumorigenesis (van de Wetering et al., 2002).

APC downregulates the transcriptional activity of β-catenin in three ways (Bienz, 2002). Its best-established function is to reduce the levels of cytoplasmic β-catenin by binding to Axin, which results in the phosphorylation of β-catenin and its subsequent proteasomal destruction. Furthermore, APC also promotes the export of nuclear β-catenin (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Finally, there are indications that APC can sequester β-catenin, thus keeping it from binding to TCF and activating transcription (Neufeld et al., 2000; Rosin-Arbesfeld et al., 2003).

In a search for additional APC-associated proteins in embryonic extracts of *Drosophila*, we identified *Drosophila* C-terminal binding protein (dCtBP). This protein and its mammalian homologs CtBP1 and CtBP2 are transcriptional corepressors that bind to a number of diverse DNA binding proteins including, apparently, some of the TCF factors (Chinnadurai, 2002). We show specific binding between APC and CtBP in vitro and specific association of these proteins in colorectal cancer cells, and we provide evidence for the functional relevance of this interaction in TCF-mediated transcription. In contrast, the in vitro binding between CtBP and TCF is very weak, and the two proteins are neither detectably associated in mammalian cells nor show any direct functional interaction with each other. Our results thus question the previously proposed role of CtBP as a direct corepressor of TCF. Rather, they suggest that CtBP sequesters nuclear APC/β-catenin complexes and antagonizes TCF-mediated transcription by lowering the availability of free β-catenin for binding to TCF.

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## Results and Discussion

### CtBP Binds to APC In Vivo and In Vitro

To identify proteins that bind to APC in *Drosophila* embryos, we incubated crude embryonic extracts with bacterially expressed *Drosophila* E-APC (Yu et al., 1999) fused to glutathione-S-transferase (GST). Analysis of associated proteins by MALDI mass spectrometry revealed dCtBP as an unexpected binding partner of E-APC. CtBP was initially discovered as a cellular protein binding to the C terminus of the adenovirus E1A protein, which suppresses its transformation potential (Boyd et al., 1993). CtBP is a transcriptional corepressor in mammals (Chinnadurai, 2002; Sollerbrant et al., 1996) and *Drosophila* (Nibu et al., 1998a, 1998b; Poortinga et al., 1998) and binds to various DNA binding proteins via a short conserved motif P-h-D-L-S-x-R/K (Schaeper et al., 1995). Mammals have a second CtBP relative, CtBP2, which also recognizes this motif (Turner and Crossley, 1998) and whose function overlaps that of CtBP (Hildebrand and Soriano, 2002).

Intriguingly, a motif similar to P-h-D-L-S-x-R/K is found in each of the 15 amino acid repeats (15R) of APC and of *Drosophila* E-APC (Figure 1A). These repeats can bind to β-catenin but cannot promote its proteasomal destruction (Rubinfeld et al., 1997), as the latter requires the Axin binding motifs of APC (e.g., von Kries et al., 2000). Therefore, there is no known function of the 15Rs in the downregulation of β-catenin. The interaction between an individual 15R and β-catenin has been characterized at the structural level (Eklof Spink et al., 2001). The presumed CtBP binding motif shares some but not all of the residues in the C-terminal half of the 15R that are engaged in the interaction with β-catenin (Figure 1A).

Binding between E-APC and dCtBP was confirmed in vitro by pull-down assays between bacterially expressed GST-dCtBP and in vitro translated E-APC (Figure 1B). This binding is comparable to that between E-APC and Armadillo (*Drosophila* β-catenin) (Figure 1B); however, Armadillo does not bind directly to GST-dCtBP

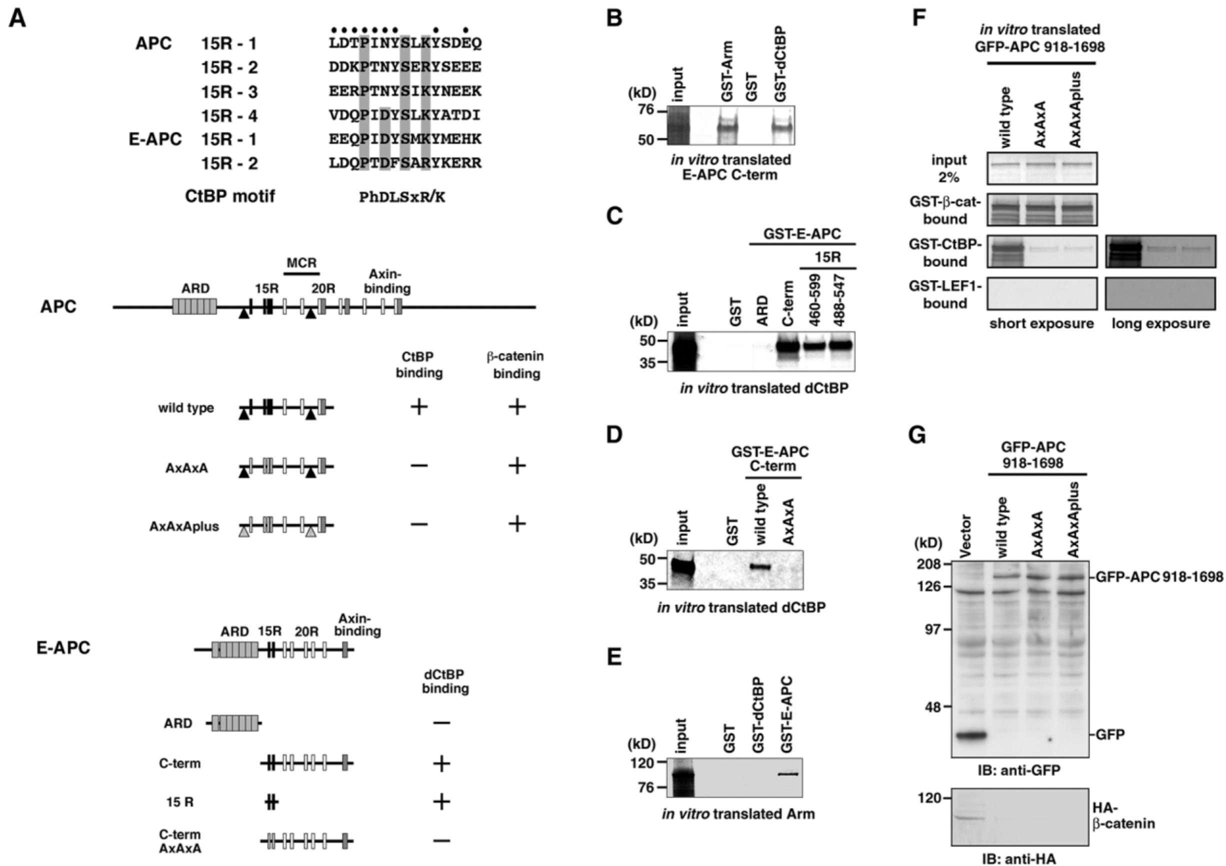

Figure 1. Direct Binding between CtBP and APC

(A) Top: CtBP binding sites within the 15Rs of APC and E-APC (dots indicate residues in 15R-1 engaged in direct contact with β-catenin). AxAxA has triple alanine substitutions in residues 1, 3, and 5 of the P-h-D-L-S-x-R/K motif (h, hydrophobic) of each 15R; AxAxAplus has further triple substitutions in two putative additional motifs (PADLAHK at codon 1001, PSDLPDS at codon 1420 of APC). Bottom: Structures of APC and E-APC, with conserved sequences boxed (ARD, gray; 15Rs, black; 20Rs, white; Axin binding sites, stippled; MCR, mutation cluster region; arrowheads point to putative additional CtBP binding sites in APC); binding activities of minimal wild-type and mutant fragments are summarized below.

(B–F) Pull-down assays between bacterially expressed GST-fusions and in vitro translated protein, as indicated; (B–E) total reactions were loaded in lanes denoted "input;" (F) 2% of each input protein is shown in the top panel; below, total reactions were loaded in each lane (long exposures of the same gels are shown on the right).

(G) Western blots of wild-type and mutant versions of GFP-APC(918–1698) or of GFP alone (top) after cotransfection with HA-β-catenin (bottom); in each case, 200 ng of plasmid was used.

(Figure 1E). A small region spanning the two 15Rs of E-APC fused to GST is sufficient for binding to in vitro translated dCtBP (Figure 1C), while a triple alanine substitution ("AxAxA") in the P-h-D-L-S motif of each 15R (in the context of the C-terminal half of E-APC) almost completely abolishes binding to dCtBP (Figure 1D). The same is true for the binding between human CtBP and a central fragment of APC (residues 918–1698) that binds efficiently to GST-CtBP, while its mutant version AxAxA binds poorly (Figure 1F). APC(918–1698) contains two further putative CtBP binding motifs that were substituted in addition ("AxAxAplus"; Figure 1A). This further reduced the binding to GST-CtBP (by >16%); no binding whatsoever was detectable with a GST-LEF-1 control (Figure 1F). Importantly, both APC mutants bind to β-catenin equally well as the wild-type (Figure 1F). Likewise, both mutants retain the ability to reduce the overall levels of coexpressed HA-tagged β-catenin in transfected *APC* mutant cancer cells (Figure 1G), though a low level of endogenous β-catenin can still be detected by immunofluoresence in these transfected cells (Figure 3D). Thus, the binding between APC and CtBP is specific and conserved and neither appears to affect APC's binding to β-catenin nor its ability to promote the destruction of cytoplasmic β-catenin.

APC is also associated with CtBP in mammalian cells: endogenous CtBP can be coimmunoprecipitated with endogenous APC, and vice versa, in 293T cells (Figure 2A) and in HCT116 colorectal cancer cells that express wild-type APC (Figure 2B). Furthermore, in *APC* mutant cancer cells, the resident APC truncations can be coimmunoprecipitated in SW480 cells, but not in COLO320 cells (Figures 2B and 2C). Notably, the 15Rs are retained only in the APC truncation of the former, but not of the

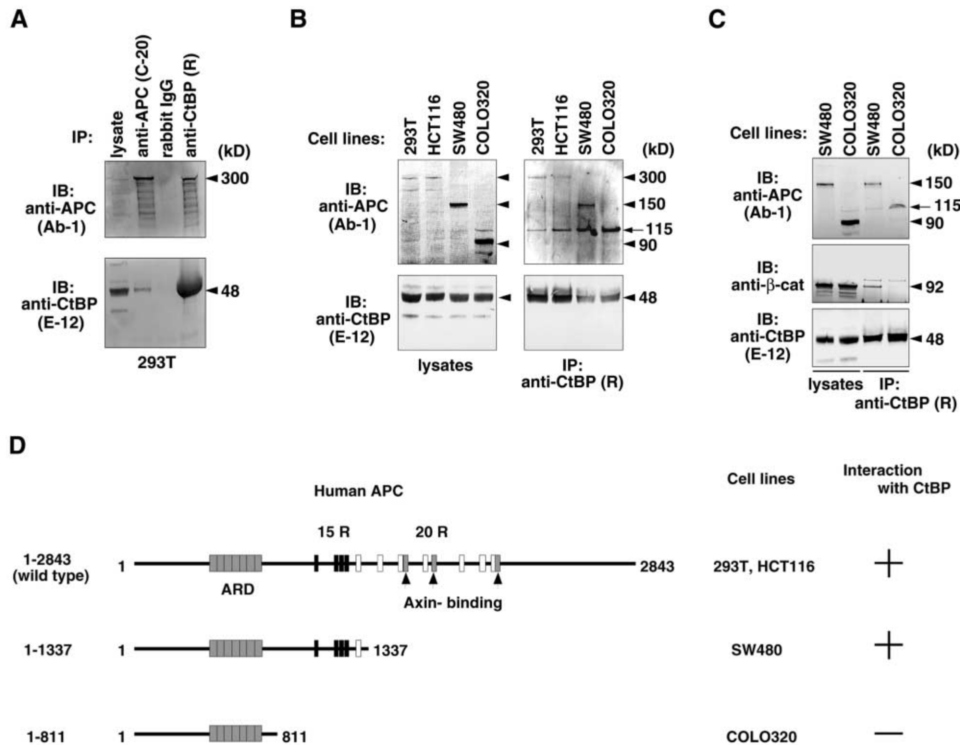


Figure 2. Association between APC and CtBP in Mammalian Cells

(A–C) Coimmunoprecipitations between endogenous CtBP and APC in various cell lines, as indicated (IP, immunoprecipitation; IB, immunoblotting; names of antibodies are given in brackets); in lanes denoted "lysate," ~3% of the total sample were loaded. Relevant bands are indicated by arrowheads (arrows in [B] and [C] point to a background band due to Ab-1, particularly prominent in COLO320 extracts). APC was also coimmunoprecipitated successfully with anti-CtBP E-12 antibody (not shown).

(D) Summary of the in vivo associations between CtBP and APC in human cells expressing wild-type APC (293T, HCT116) or APC truncations (SW480, COLO320).

latter (Figure 2D). Thus, the association of APC with CtBP in mammalian cells depends on its 15Rs.

### Mutant APC that Cannot Bind to CtBP Is Less Active in Complementation Assays of Colorectal Cancer Cells

Few colorectal carcinomas express APC truncations that lack the 15Rs (e.g., Miyaki et al., 1994). COLO320 is one of the rare colorectal cancer cell line of this type (Rowan et al., 2000). Interestingly, this line exhibits exceptionally high TCF-mediated transcription (Rosin-Arbesfeld et al., 2003). This suggests that the 15Rs may harbor an activity that is critical for the downregulation of the transcriptional activity of TCF.

To test whether the binding of CtBP to the 15Rs is functionally relevant, we used a complementation assay of *APC* mutant cancer cells based on a luciferase reporter linked to TCF binding sites (pTOPFLASH; Korinek et al., 1997). This quantitative assay is highly specific for TCF-mediated transcription and serves as a fairly direct readout of exogenous APC function in restoring low levels of TCF transcription. COLO320 cells show very high TOPFLASH values (Figure 3A), $>2\times$ higher than those of SW480 cells and up to $5\times$ higher than those of other *APC* mutant colorectal cancer cells (Rosin-Arbesfeld et al., 2003). These values are reduced substantially (Figure 3A) after cotransfection with APC(918–1698), which spans the 15Rs and the 5′-most nuclear export signal (NES1506) and Axin binding site (Figure 1A). Similar APC fragments have previously been found to efficiently reduce the β-catenin levels in SW480 cells (Munemitsu et al., 1995). In contrast, the AxAxA mutant is less active in reducing TOPFLASH values, and AxAxAplus is even less active (Figure 3A). The control values of pFOPFLASH (containing mutant TCF sites; Korinek et al., 1997) are low and unchanged by the mutants (Figure 3A). We conclude that the binding between APC and CtBP is critical for the APC-mediated downregulation of the transcriptional activity of β-catenin. The residual activities of AxAxA and AxAxAplus in this assay are likely to reflect their ability to promote Axin-mediated destruction (Figure 1G) and nuclear export of β-catenin (see above; note that APC(918–1698) and its mutant versions shuttle in and out of the nucleus, as judged by their nuclear accumulation after exposure to leptomycin B; Figure 3D).

### Evidence that CtBP Sequesters APC/β-Catenin Complexes and Diverts Free Nuclear β-Catenin away from TCF

Previous evidence indicated that APC can sequester nuclear β-catenin and keep it from binding to TCF and

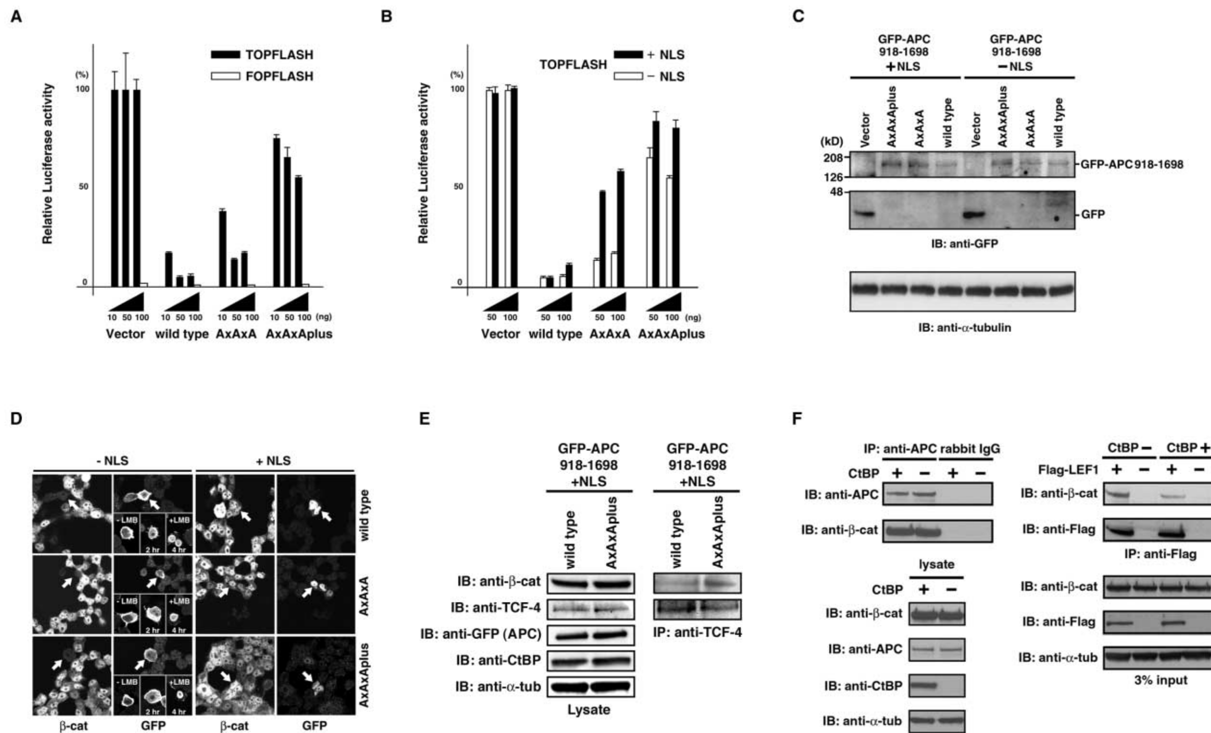


**Figure 3. Binding between APC and CtBP Is Required for Reduction of TCF-Mediated Transcription**

(A and B) TOPFLASH assays, measuring complementation of COLO320 cells by wild-type and mutant GFP-APC(918–1698) with or without NLS, as indicated; average relative luciferase values from 3–5 experiments are given (error bars indicate standard deviations; FOPFLASH control values are given in [A]).

(C) Western blots of total cell extracts, after transfection with GFP or of GFP-APC(918–1698) plasmids, probed with anti-GFP (see also Figure 1G) or with anti-α-tubulin as internal control.

(D) COLO320 cells, transfected with wild-type and mutant GFP-APC(918–1698) plasmids as in (B) and stained as indicated. In the absence of an NLS, these APC proteins are predominantly cytoplasmic, but low nuclear levels are also detected (arrows), which are significantly increased after LMB treatment (inserts). Linkage to an NLS efficiently targets APC proteins and, consequently, β-catenin to the nucleus (arrowheads).

(E) Right: Coimmunoprecipitation of endogenous β-catenin with endogenous TCF-4 in COLO320 cells transfected with wild-type (left) or AxAxAplus mutant NLS-APC(918–1698) (right) after cell sorting. Left: total protein levels in lysates. Quantitative analysis of three independent experiments shows 1.5×–2× higher levels of TCF-4 bound β-catenin in the presence of the APC mutant compared to the wild-type.

(F) Top: Coimmunoprecipitation of endogenous β-catenin with endogenous APC (left) or Flag-LEF-1 (right) in *CtBP1*$^{-/-}$ *CtBP2*$^{-/-}$ mutant mouse cells or in parental control cells, showing 2×–3× higher levels of LEF-1-associated β-catenin in the mutants (quantitative measurements from four independent experiments). The total levels of β-catenin, APC, and of the APC bound β-catenin are the same in the two cell lines.

activating transcription (Neufeld et al., 2000; Rosin-Arbesfeld et al., 2003). This sequestration can be demonstrated experimentally if an APC fragment is targeted to the nucleus by linkage to a nuclear localization signal (NLS): this causes a dramatic nuclear accumulation of endogenous β-catenin, but these high levels of nuclear β-catenin are ineffective in stimulating TCF-mediated transcription (Figure 3B; Rosin-Arbesfeld et al., 2003). This therefore provides an assay for measuring the sequestration of nuclear β-catenin by APC.

We thus tested NLS-fusions of the AxAxA and AxAxAplus mutants in this sequestration assay. Interestingly, the mutant NLS-fusions are less active in reducing TOPFLASH values than their wild-type controls (Figure 3B). These differences are significant since the expression levels of wild-type and mutant NLS-fusions are essentially the same (Figure 3C). Notably, the loss of function of the AxAxA and AxAxAplus mutants in reducing β-catenin activity is exacerbated in this sequestration assay where the levels of nuclear β-catenin are high (Figure 3B). This suggests a role of the APC-CtBP interaction in sequestering nuclear β-catenin.

A possible model is that APC binds to free nuclear β-catenin in competition with TCF and targets β-catenin to CtBP (by being an adaptor between these two proteins), thus diverting β-catenin away from TCF. CtBP, being anchored at specific sites within the nucleus (Kagey et al., 2003; Lin et al., 2003), could act as a "sink" for APC/β-catenin complexes, thus shifting the binding equilibrium of β-catenin yet further away from TCF.

Three lines of evidence support this model. First, β-catenin can be detected in a complex with CtBP in SW480, but not in COLO320 cells (Figure 2C), whose APC truncation can bind neither CtBP nor β-catenin. Second, in COLO320 cells transfected with NLS-fusions of APC, we estimate that the levels of endogenous TCF-4/β-catenin complexes are 1.5×–2× higher in the case of AxAxAplus compared to the wild-type control (Figure 3E). These increased levels of TCF-4/β-catenin complexes are likely to be the basis for the high TCF-mediated transcription in the complementation assays (Figure 3B). Third, in *CtBP* mutant mouse cells (Hildebrand and Soriano, 2002) expressing tagged LEF-1, 2×–3×

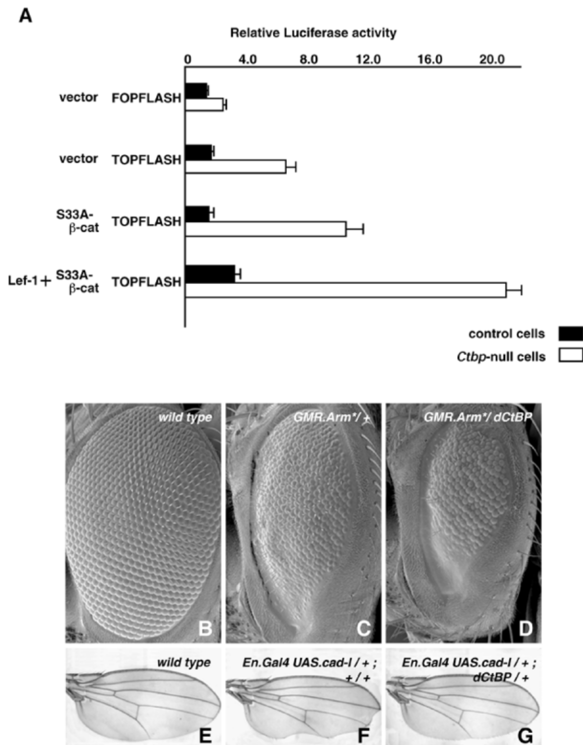


Figure 4. Loss of CtBP Increases the Activity of β-Catenin or Armadillo

(A) TOPFLASH assays in *CtBP* mutant or control cells, cotransfected with control GFP or with activated β-catenin (S33A), + or − mouse Lef-1 (200 ng of each plasmid), as indicated (internal control pRL-CMV); control FOPFLASH values are also given.

(B–G) Fly eyes and wings, with genotypes as indicated. Heterozygosity for dCtBP enhances the rough eye phenotype (D, compare to C) due to activated Armadillo (F76), but suppresses the wing nick phenotype in the posterior margin (G, compare to F) due to Armadillo depletion (En.Gal4 UAS.cad-I).

more endogenous β-catenin can be coimmunoprecipitated with LEF-1 than in the corresponding parental control cells (heterozygous for both alleles) (Figure 3F, right panels). The total levels of β-catenin are the same in the two cell lines, as are the amounts of APC bound β-catenin (Figure 3F, left panels). The latter two lines of evidence indicate that CtBP reduces the availability of β-catenin for binding to TCF.

If so, absence of CtBP should result in elevated levels of TCF-mediated transcription. Indeed, the basal TOPFLASH activity (due to endogenous TCF/β-catenin) in *CtBP* mutant cells is increased ~3.7× compared to their control cells (Figure 4A). Furthermore, cotransfection of activated β-catenin (S33A mutant) and Lef-1 stimulate TOPFLASH activity to higher levels in *CtBP* mutant cells compared to the control (Figure 4A). By comparison, we detect <2× differences in transcriptional activity between mutant and wild-type cells if FOPFLASH or an SV40-based control reporter (pRL-SV) are tested (Figure 4A). Indeed, the activity levels of the internal control renilla reporter (pRL-CMV) are the same in both cell lines (not shown). Therefore, Lef-1-mediated transcription is more sensitive to CtBP loss than the transcription mediated by other transcription factors. Thus, CtBP appears to antagonize TCF-mediated transcription in a relatively specific way.

## CtBP Interacts Neither Physically nor Functionally with TCF in Mammalian Cells

It has been reported that *Xenopus* CtBP can bind to XTcf-3 and antagonize the transcription of TCF target genes in the early *Xenopus* embryo (Brannon et al., 1999). These authors noted that TCF-3 and TCF-4 factors possess CtBP binding motifs and suggested that CtBP may be a corepressor of these TCFs (Brannon et al., 1999). Potentially, this could explain the increased basal levels of TCF-mediated transcription in *CtBP* mutant cells compared to their parental controls (Figure 4A, second and third columns). However, it is unlikely to explain the increased levels of Lef-1-stimulated transcription (Figure 4A, fourth columns), given that Lef-1 is a TCF factor that lacks CtBP binding motifs (Brannon et al., 1999; Hovanes et al., 2000).

In vivo association between CtBP and TCF had never been demonstrated, so we decided to examine this in comparison to the in vivo association between CtBP and APC (Figure 2). First, we asked whether endogenous CtBP and TCF-4 coimmunoprecipitate in colorectal cancer cells, given that TCF-4 is expressed in these cells (Korinek et al., 1997). β-catenin coimmunoprecipitates with TCF-4, as expected; however, CtBP is not detectable in the same TCF-4 immunoprecipitate (Figure 5A, left panel, lane 4). Conversely, while APC coimmunoprecipitates with CtBP (see also Figures 2B and 2C), TCF-4 does not (Figure 5A, left panel, lane 2; note that the antibody used in this case would have also detected CtBP-associated TCF-3; see Experimental Procedures). Thus, endogenous CtBP is associated with APC, but not with TCF, in colorectal cancer cells. Notably, the same is true in 293T cells in which TCF is transcriptionally inactive: endogenous CtBP is associated with APC and β-catenin, but not with endogenous TCF-4 (Figure 5A, right panel, lane 4). We conclude that TCF is not detectable in a complex with CtBP, regardless of cell type and transcriptional activity.

It was previously reported that exogenous TCF-4 can repress TOPFLASH transcription in transfected simian COS cells (that lack E1A expression) in a CtBP-dependent manner, while a C-terminal truncation of TCF-4 without the CtBP binding motifs (such as those arising from frameshift mutations in *TCF-4* in some microsatellite-unstable colorectal carcinomas; Ruckert et al., 2002) does not respond to overexpressed CtBP in this assay (Valenta et al., 2003). We repeated these experiments by comparing the activities of mutant TCF-4, whose two CtBP binding motifs were mutated in the same way as those of APC (TCF-4 AxAxA) and its wild-type control in TOPFLASH assays, and in their response to overexpressed CtBP. We confirmed that overexpressed TCF-4 can repress TOPFLASH transcription in a dose-dependent manner in transfected SW480 and COS cells (Figure 5B, black bars; not shown). However, the AxAxA TCF-4 mutant was similarly inhibitory (Figure 5B, white bars), despite being expressed at slightly higher levels than wild-type TCF (especially at low doses of transfected plasmid; Figure 5C). Furthermore, the mutant was equally responsive to coexpressed CtBP as the wild-type TCF-4 (Figure 5D). Therefore, although the AxAxA

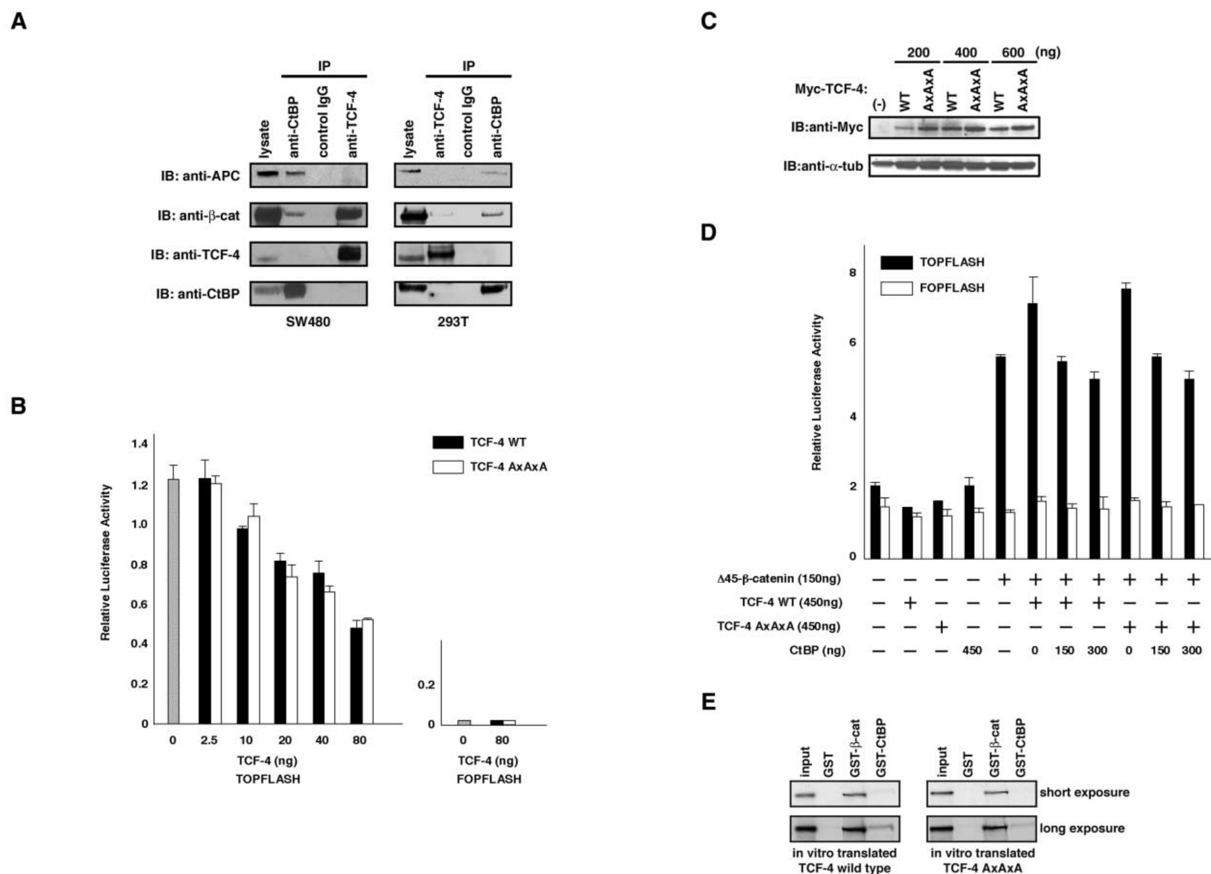


Figure 5. Lack of Physical or Functional Interactions between CtBP and TCF in Mammalian Cells

(A) Coimmunoprecipitations between endogenous CtBP and APC or TCF-4 and between endogenous TCF-4 and CtBP or β-catenin, in SW480 (left) or 293T cells (right), as indicated (IP, immunoprecipitation; IB, immunoblotting; lane 1, ~3% of the total sample were loaded; lane 3, IgG was used for control immunoprecipitations). Note that TCF-4 fails to immunoprecipitate with CtBP, although APC (and β-catenin) do so (see also Figures 2B and 2C); conversely, CtBP (and APC) fail to immunoprecipitate with TCF-4, although β-catenin does so.

(B) TOPFLASH assays in SW480 cells, transfected with control GFP ("0") or with increasing amounts of TCF-4 or TCF-4 AxAxA (internal controls pRL-CMV); control FOPFLASH values are given on the right.

(C) Expression levels of wild-type and mutant AxAxA TCF-4 at different plasmid doses.

(D) TOPFLASH assays in COS cells, as in (B), cotransfected with or without 150 ng of activated β-catenin (Δ45-β-catenin), 450 ng of TCF-4 or TCF-4 AxAxA, and increasing amounts of GFP-CtBP as indicated. TCF-4 AxAxA is similarly active in repressing transcription (B) and responds equally to coexpressed CtBP as wild-type TCF-4 (D), despite being expressed at slightly higher levels ([C]; note that the luciferase values in [B] and [D] have not been corrected for TCF expression levels).

(E) Pull-down assays between bacterially expressed CtBP or β-catenin and in vitro translated wild-type or AxAxA mutant TCF-4, as indicated; 7% of total reactions were loaded in lanes denoted "input." A long exposure is shown at the bottom to visualize the weak binding between CtBP and TCF-4.

mutation affects the activity of APC(918–1698) in TCF-specific transcription assays (Figures 3A and 3B), the same mutation in TCF-4 does not affect its activity in these assays (Figures 5B and 5D). In agreement with this, a comparable double mutation of the CtBP binding motifs in XTcf-3 does not reduce its repressive potential in *Xenopus* embryos (Brannon et al., 1999). Note that this double mutation does reduce the in vitro binding of XTcf-3 to CtBP (Brannon et al., 1999), and so does the AxAxA double mutant of TCF-4 (Figure 5E). However, the in vitro binding between CtBP and TCF-4 is ~10× less strong than that between TCF-4 and β-catenin (Figure 5E). Thus, the in vitro binding between CtBP and TCF, although apparently specific, is very weak indeed. It may be spurious, given the lack of a detectable association between these proteins in vivo.

In summary, we were unable to obtain any evidence for a significant physical or functional interaction between CtBP and TCF. Our results thus question the idea that CtBP functions generally as a corepressor of TCF factors. We agree with Ruckert et al. (2002) who concluded that the TCF-4 frameshift mutations observed in microsatellite-unstable colorectal carcinomas are passenger mutations without any functional relevance for TCF-mediated transcription or tumorigenesis.

## Antagonism between CtBP and Activated Armadillo during Development

We asked whether dCtBP might antagonize Armadillo-mediated transcription during *Drosophila* development. However, this is not straightforward to test, since *dCtBP* mutants show highly pleiotropic mutant phenotypes:

null mutant embryos are grossly abnormal and do not develop beyond early stages, due to failing interactions between dCtBP and segmentation gene products (Nibu et al., 1998a, 1998b; Poortinga et al., 1998). This precludes a meaningful analysis of dTCF target gene expression in these mutants (E. Saller and M.B., unpublished results). And although dCtBP has been implicated in antagonizing dTCF transcription in the developing midgut, this is an indirect effect mediated by the DNA binding protein Brinker to which CtBP can bind (Saller et al., 2002). Likewise, CtBP loss in the mouse causes pleiotropic mutant phenotypes, one of which—unexpectedly—mimics loss of Wnt signaling (Hildebrand and Soriano, 2002), but this could also be an indirect effect of CtBP binding to another target protein outside the Wnt pathway.

Thus, to explore the regulatory relationship between dCtBP and Armadillo during development, we asked whether dCtBP loss would affect the phenotypic consequences of overactive or depleted Armadillo. This is the case: lowering the dose of dCtBP enhances the rough eye phenotype caused by activated Armadillo (Figures 4B–4D; Freeman and Bienz, 2001), but the same condition suppresses the wing nick phenotype due to Armadillo depletion in cells whose stimulation by Wingless is required for normal wing margin formation (Figures 4E–4G; Sanson et al., 1996). These genetic interactions are similar to those of negative components of the Wnt pathway that downregulate Armadillo, such as *Drosophila* Axin and APC (Freeman and Bienz, 2001), consistent with dCtBP antagonizing Armadillo. Again, we emphasize that this antagonism is unlikely to be due to dCtBP being a direct corepressor of dTCF, given that the latter does not contain any CtBP binding motifs (Brannon et al., 1999). Our results suggest that the antagonism between CtBP and Armadillo/β-catenin is conserved and operates in multiple tissues and cell types.

## Conclusions

We presented evidence that CtBP binds to APC directly and specifically via the conserved 15Rs of APC and that the association of the two proteins in vivo is functionally relevant as it is required for the full activity of APC in reducing TCF-mediated transcription in colorectal cancer cells. In contrast, we failed to obtain any evidence for a direct physical or functional interaction between CtBP and TCF in mammalian cells, calling into question whether CtBP acts generally as a transcriptional corepressor of TCF factors.

Instead, our evidence suggests that CtBP antagonizes TCF-mediated transcription by cooperating with APC to sequester nuclear β-catenin. This sequestration could be a safeguard function of APC, operating in parallel to (and to some extent redundantly with) its other functions in promoting nuclear export and degradation of β-catenin. We propose that APC sequesters β-catenin by targeting it to CtBP, thus lowering the pool of free nuclear β-catenin that is available for binding to TCF. The sequestration of the APC/β-catenin complex by CtBP may be based on spatial segregation within the nucleus (e.g., anchoring of the complex at specific subnuclear bodies; Kagey et al., 2003; Lin et al., 2003). Whatever the precise mechanism, the observed functional cooperation between CtBP and APC in colorectal cancer cells suggests a role of CtBP as a tumor suppressor in the colon.

## Experimental Procedures

### Plasmids, Mutagenesis, and In Vitro Pull-Down Assays

Plasmids encoding *Drosophila* E-APC(453–1067) and dCtBP have been described (Saller et al., 2002; Yu et al., 1999). Plasmids encoding human CtBP1, APC(918–1698), β-catenin(134–671), TCF-4, and *Drosophila* Armadillo(156–690) and E-APC (2–455, 460–599, and 488–547) were generated by standard PCR cloning procedures and checked by sequencing; human CtBP1 was tagged with GFP by subcloning into pEGFP-C2. Full-length human β-catenin was tagged at its N terminus with a triple HA tag and subcloned into pcDNA3.1 (Invitrogen Life Technologies). Flag-tagged human LEF-1 (kindly provided by D. Ayer) was subcloned into the HpaI site of the pMIG retroviral vector (Van Parijs et al., 1999). Myc-tagged human TCF-4 (in pcDNA1) (Korinek et al., 1997) was kindly provided by F. Townsley.

Triple alanine substitutions were introduced into fragments of APC(918–1698) and E-APC(453–1067), and into TCF-4, by using a site-directed mutagenesis kit (Stratagene); details of the primers are available on request.

Wild-type and mutant APC(918–1698) were subcloned into pEGFP-C2 (CLONTECH Laboratories) and into pEGFP-C2+NLS (between EcoRI and BamHI), a vector containing an NLS from SV40 large T antigen (TPPKKKRKVED, between XhoI and EcoRI) (Rosin-Arbesfeld et al., 2003), between their EcoRI and BamHI sites. Each NheI/BamHI fragment (including its N-terminal GFP tag), and also full-length TCF-4, were subcloned into pBluescript II (between SpeI and BamHI) to generate $^{35}$S-labeled proteins by the coupled transcription-translation TNT system (Promega).

Human CtBP, β-catenin, and LEF-1(1–110) and *Drosophila* Armadillo and E-APC fragments were also subcloned into pGEX-2T or pGEX-4T1 (Amersham Pharmacia Biotech). Recombinant GST-tagged proteins were expressed in *Escherichia coli* BL21 and purified by glutathione-Sepharose (Amersham Pharmacia Biotech). In vitro pull-down assays were performed as described (Hamada et al., 1999); same amounts of GST-fusions were used, as confirmed by Coomassie blue staining. Quantitative analysis was done with a Personal Densitometer SI (Molecular Dynamics); in Figure 1F, input protein values (top) were taken into account, but background values were not subtracted, given the low signals of the GST-CtBP bound APC mutants.

### Identification of E-APC Binding Proteins

Cytoplasmic extracts of dechorionated *Drosophila* embryos (0–12 hr), generated as described previously (Soeller et al., 1988), were kindly provided by C.P. Verrijzer (Leiden University Medical Center). Isolation of E-APC binding proteins was performed essentially as described (Soeller et al., 1988).

Molecular mass analyses of tryptic fragments were kindly performed by S. Peak-Chew and F. Begum. Samples were analyzed with a Voyager-DE-STR matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (PerSeptive Biosystem). Proteins were obtained by database searches using peptide masses with the Mascot program (http://www.matrixscience.com). Molecular weights of 13 tryptic peptides derived from a 40 kDa protein purified specifically with GST-E-APC(453–1067), but not with GST or GST-E-APC(2–455), coincided with peptides from dCtBP.

### Coimmunoprecipitations and Western Blotting

For coimmunoprecipitations, cells were pelleted and lysed in extraction buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, Protease Inhibitor cocktail; Roche). Lysates were centrifuged at 15,000 rpm at 4°C for 20 min and the supernatants were incubated with protein G-Agarose beads (Roche) for 1 hr at 4°C. The mixture was then centrifuged for 10 s at 15,000 rpm, and the supernatant was transferred to a fresh tube and incubated with the appropriate antibody for 1 hr at 4°C. Protein G-Agarose was then added and incubated for 30 min at 4°C. The mixture was centrifuged for 10 s at 15,000 rpm, the supernatant was removed, and the precipitate was washed 4× with 1 ml of extraction buffer. After boiling in SDS-sample buffer, the immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon-P (Millipore) and immunoblotted with the appropriate antibodies followed by ProtoBlot II AP System (Promega) (Hamada and Bienz, 2002) or by

the enhanced chemiluminescence (ECL) Western blotting system (Amersham) (Rosin-Arbesfeld et al., 2003). Comparative quantitative analysis by densitometry revealed that the values obtained for the same samples subjected in parallel to the two different blotting methods were essentially the same.

The following antibodies were used for immunoprecipitation: anti-APC rabbit polyclonal IgG (1:50) (C-20; Santa Cruz Biotechnology); anti-CtBP mouse monoclonal IgG (1:50) (E-12; Santa Cruz Biotechnology) or anti-CtBP rabbit antiserum (1:100) (Schaeper et al., 1995) (kindly provided by G. Chinnadurai); and anti-TCF-4 (1:500) (clone 6H5-3, Upstate). Anti-Flag M2 affinity gel (Sigma) was used for immunoprecipitation of Flag-LEF-1. The following antibodies were used for immunoblotting: anti-APC mouse monoclonal IgG (1:100) (Ab-1; Oncogene Research Products); anti-CtBP mouse monoclonal IgG (1:200) (E-12) or anti-CtBP rabbit antiserum (1:2000) (Schaeper et al., 1995); anti-β-catenin mouse monoclonal IgG (1:1000) (C19222; Transduction Laboratories); anti-TCF-3/4 (1:250) (6F12-3; Upstate); anti-α-tubulin mouse monoclonal IgG (1:1000) (Sigma); horseradish peroxidase-conjugated anti-Flag mouse monoclonal IgG M2 (1:1000) (Sigma); and anti-myc mouse monoclonal IgG (1:1000) (9E10.2; Invitrogen).

### Cell Transfections and Luciferase Assays

Human 293T, HCT116, SW480, and COLO320 and mouse CtBP1$^{-/-}$ CtBP2$^{-/-}$ mutant and parental control cells (CtBP1$^{+/-}$ CtBP2$^{+/-}$) (Hildebrand and Soriano, 2002) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with LipofectAMINE (Invitrogen Life Technologies); unless indicated otherwise, 100 ng of total DNA was used per well of a 6-well plate. For Figure 3E, COLO320 cells were transfected with Lipofectamine 2000 (Invitrogen Life Technologies), and cells expressing GFP-tagged NLS-fusions (∼10%) were sorted by a fluorescence-activated cell sorter and subsequently used for coimmunoprecipitations. For Figure 3F, mouse cells were infected with retroviral particles. Ecotropic retroviral supernatants were generated by transfection of Phoenix packaging cells (G.P. Nolan, Stanford University) with pMIG-Flag-LEF-1 or pMIG by standard calcium phosphate precipitation. 48 hr after transfection, supernatants were harvested and used for infection (in the presence of 4 μg/ml polybrene).

To assay TCF-mediated transcription, pTOPFLASH and pFOPFLASH (Korinek et al., 1997) and pRL-CMV and pRL-SV (encoding renilla) were used; relative luciferase values (using pRL-CMV as internal control) were determined with the Dual Luciferase Reporter assay system (Promega) as described (Rosin-Arbesfeld et al., 2000). To stimulate pTOPFLASH, pEGFP-C1-S33A human β-catenin (provided by R. Rosin-Arbesfeld) or Flag-tagged Δ45-β-catenin (in pcDNA3.1; Townsley et al., 2004), and mouse Lef-1 (pJS55, provided by F. Townsley) were cotransfected. In Figures 5B and 5D, TOPFLASH values are shown for a medium dose of transfected TCF-4 plasmids (450 ng), but the results were essentially the same at higher and lower doses (600 ng and 300 ng, respectively).

Expression and subcellular localization of GFP-tagged APC were monitored by immunofluorescence (anti-GFP rabbit antiserum) (1:5000) and by Western blotting (anti-GFP mouse monoclonal IgG; B-2, Santa Cruz Biotechnology) (1:500), as described (Rosin-Arbesfeld et al., 2001). Inhibition of nuclear export (Kudo et al., 1998) with leptomycin B (50 ng/ml) was carried out 48 hr after transfection, as described (Rosin-Arbesfeld et al., 2000). Expression of β-catenin was monitored with an anti-β-catenin monoclonal IgG (see above) (1:500).

For the experiment in Figure 1G, COLO320 cells were transfected with pEGFP-C2-APC(918–1698) along with pcDNA3.1-HA-tagged β-catenin and pRL-CMV. Cell lysates were subjected to immunoblotting analysis with anti-HA rat IgG (3F10; Roche) (1:250). The amounts of lysates used for Western blotting were normalized by measuring renilla luciferase activities of cotransfected pRL-CMV.

### Fly Genetics

Transformant lines F76 (GMR.Arm*; Freeman and Bienz, 2001) and engrailed.GAL4 UAS.cad-I (Sanson et al., 1996) were crossed with *yw* or *dCtBP$^{87De-10}$/TM3* at 25°C. Flies were prepared for scanning electron microscopy as described (except that flies were directly mounted for spraying, without dehydration in ethanol), and wings were mounted in Euparal for viewing under bright-field (Barker et al., 2001).

### Acknowledgments

We thank P. Verjizer for *Drosophila* extract and technical advice, T. Tanaka and T. Rabbitts for providing CtBP mutant cells overexpressing LEF-1 and for advice with cell sorting, G. Chinnadurai for the CtBP antibody, J. Hildebrand for the *CtBP* mutant cell line, M. Crossley, D. Ayer, R. Rosin-Arbesfeld, and F. Townsley for plasmids, S. Peak-Chew and F. Begum for mass spectrometry analysis, and R. Rosin-Arbesfeld for critical comments on the manuscript.

Received: October 15, 2003
Revised: August 20, 2004
Accepted: August 23, 2004
Published: November 8, 2004

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