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The oligomeric state of CtBP determines its role as a transcriptional co-activator and co-repressor of Wingless targets

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C-terminal-binding protein (CtBP) is a well-characterized transcriptional co-repressor that requires homo-dimerization for its activity. CtBP can both repress and activate Wingless nuclear targets in *Drosophila*. Here, we examine the role of CtBP dimerization in these opposing processes. CtBP mutants that cannot dimerize are able to promote Wingless signalling, but are defective in repressing Wingless targets. To further test the role of dimerization in repression, the positions of basic and acidic residues that form inter-molecular salt bridges in the CtBP dimerization interface were swapped. These mutants cannot homo-dimerize and are compromised for repression. However, their co-expression leads to hetero-dimerization and consequent repression of Wingless targets. Our results support a model where CtBP is a gene-specific regulator of Wingless signalling, with some targets requiring CtBP dimers for inhibition while other targets utilize CtBP monomers for activation of their expression. Functional interactions between CtBP and Pygopus, a nuclear protein required for Wingless signalling, support a model where monomeric CtBP acts downstream of Pygopus in activating some Wingless targets.

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Introduction

Wnt/β-catenin signalling has crucial roles in many aspects of embryonic development and adult homeostasis (Cadigan and Nusse, 1997; Logan and Nusse, 2004; Clevers, 2006; Cadigan, 2008). Misregulation of this pathway is causal for several different cancers and other diseases (Giles *et al*, 2003; MacDonald *et al*, 2009). This pathway is activated by a highly conserved group of secreted glycolipoproteins called Wnts, which promote the stabilization and nuclear translocation of cytosolic β-catenin (Kikuchi *et al*, 2006; Cadigan and Peifer, 2009; MacDonald *et al*, 2009). Members of the T-cell factor (TCF)/lymphoid enhancer factor-1 family of transcription factors are major nuclear binding partners of β-catenin. Given the widespread importance of Wnt/β-catenin signalling in normal and pathological states, elucidating how the pathway regulates target gene expression through TCFs remains an important goal in furthering our understanding of Wnt biology.

In the absence of Wnt signalling, several different modes of regulation operate to repress target gene expression. Although β-catenin is constantly synthesized, it is also constitutively subjected to phosphorylation by a protein complex, which includes Axin, adenomatous polyposis coli (APC), casein kinase I and glycogen synthase kinase 3. Phosphorylated β-catenin is then ubiquitinated and subjected to proteosome-mediated degradation (Kikuchi *et al*, 2006; Kennell and Cadigan, 2009). β-Catenin that escapes this destruction is prevented from binding to TCFs by several factors, which bind to either TCF or β-catenin and/or promote nuclear efflux of β-catenin (Takemaru *et al*, 2003; Hamada and Bienz, 2004; Parker *et al*, 2007). These factors serve to set the threshold of nuclear β-catenin needed to affect gene regulation. Finally, many Wnt transcriptional targets are repressed in the absence of signalling by TCFs in conjunction with co-repressors. TCF-mediated recruitment of β-catenin to Wnt-regulated elements (WREs) causes a 'transcriptional switch' of TCF from a repressor to an activator, turning on Wnt target gene expression (Cadigan and Peifer, 2009; Mosimann *et al*, 2009).

Many factors have been reported to contribute to TCF-mediated repression of WREs in the absence of signalling and TCF-mediated activation of WREs upon stimulation of the pathway. Negative regulators include the co-repressor TLE/Groucho, the transcriptional repressor Kaiso and the Brahma and ACF chromatin remodelling complexes (Cavallo *et al*, 1998; Roose *et al*, 1998; Collins and Treisman, 2000; Park *et al*, 2005; Liu *et al*, 2008). These factors are either physically displaced or somehow counteracted upon β-catenin binding to TCFs (Daniels and Weis, 2005; Parker *et al*, 2007; Liu *et al*, 2008). β-Catenin then recruits many co-activators to WREs, for example, the Legless (Lgs)–Pygopus (Pygo) complex to the N-terminal transactivation domain of β-catenin, and CBP/p300 and Paraformbin/Hyrax to the β-catenin's C-terminal transactivation domain (Hecht *et al*, 2000; Stadeli and Basler, 2005; Mosimann *et al*, 2006; Li *et al*, 2007).

C-terminal-binding protein (CtBP) is another factor that has been shown to have important roles in modulating the Wnt/β-catenin pathway. Overexpression of CtBP can inhibit Wnt signalling (Brannon *et al*, 1999; Valenta *et al*, 2003; Hamada and Bienz, 2004; Fang *et al*, 2006). Consistent with CtBP acting as a transcriptional co-repressor in many contexts (Turner and Crossley, 2001; Chinnadurai, 2007), CtBP has been reported to bind directly to TCFs (Brannon *et al*, 1999;

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Valenta *et al*, 2003). However, more recent reports have been unable to find a detectable interaction (Hamada and Bienz, 2004; Valenta *et al*, 2006). Instead, a CtBP–APC complex was shown to bind to β-catenin, and prevent its interaction with TCF4, thus blocking Wnt target gene activation (Hamada and Bienz, 2004; Sierra *et al*, 2006).

Our laboratory has previously shown that in *Drosophila* cells, CtBP is required for repression of several Wingless (Wg, a fly Wnt) targets. CtBP is enriched at the WREs of one of these targets, in a similar pattern as TCF (Fang *et al*, 2006). However, CtBP recruitment to these WREs is not dependent on TCF (Fang *et al*, 2006). In addition, several Wg targets were not repressed by CtBP, but instead required CtBP for maximal activation by the pathway. CtBP was recruited to the WRE of one of these targets in a TCF and Armadillo (Arm, the fly β-catenin)-dependent manner (Fang *et al*, 2006). Furthermore, a Gal4–Arm fusion requires CtBP for activation of a *UAS-luc* reporter and Gal4–Arm can recruit CtBP to the reporter gene chromatin (Fang *et al*, 2006). Thus, CtBP contributes to both aspects of the TCF transcriptional switch, in a gene-specific manner.

The CtBP family of proteins all contain a conserved central domain with high homology to NAD^+/NADH-dependent dehydrogenases (Kumar *et al*, 2002; Nardini *et al*, 2003). Dehydrogenase activity has been detected in recombinant human CtBP1 (hCtBP1) (Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Achouri *et al*, 2007) but the role of catalytic function in the transcriptional activity of CtBP is controversial. Mutations in the catalytic site compromise co-repressor activity (Kumar *et al*, 2002; Zhang and Arnosti, 2011), although not in all contexts (Phippen *et al*, 2000; Grooteclaes *et al*, 2003; Sutrias-Grau and Arnosti, 2004; Mani-Telang *et al*, 2007; Kuppuswamy *et al*, 2008). The catalytic activity of CtBP is crucial for a complete rescue of CtBP mutants in *Drosophila* (Zhang and Arnosti, 2011). However, the role of CtBP in potentiating the activity of Gal4–Arm in fly cells does not require dehydrogenase activity (Fang *et al*, 2006).

Another important factor that can affect the transcriptional activity of the CtBP family of proteins is their quaternary structure. In cells, CtBP is thought to exist in an equilibrium between monomers (Kim *et al*, 2005; Zhao *et al*, 2009), homo-dimers and possible higher order structures (Balasubramanian *et al*, 2003; Shi *et al*, 2003; Thio *et al*, 2004; Kim *et al*, 2005; Mani-Telang *et al*, 2007; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009). Dimerization is stimulated by NAD^+/NADH binding (Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Kim *et al*, 2005; Kuppuswamy *et al*, 2008; Nardini *et al*, 2009), but mutations in NAD^+-binding domain do not abolish dimerization in all cases (Thio *et al*, 2004; Mani-Telang *et al*, 2007). When crystallized, mammalian CtBP proteins exist as dimers, and the dimerization interface has been well defined (Kumar *et al*, 2002; Nardini *et al*, 2003). Mutations in the dimerization interface have been shown to reduce the function of CtBP as a co-repressor in several contexts (Kumar *et al*, 2002; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009).

In this report, we examine whether dimerization of CtBP has a role in mediating the Wg/Wnt transcriptional switch in fly cells. Mutant forms of CtBP that cannot dimerize are still able to activate Wg targets, but are no longer capable of repression. However, co-expression of different monomeric forms of CtBP that can hetero-dimerize restores the repression activity. We conclude that CtBP dimers act in repression of Wg targets while CtBP monomers function in transcriptional activation of Wg targets. In the activation of Wg targets, functional interactions between CtBP and pygo in cell culture and the developing fly wing support a model where monomeric CtBP acts downstream of Pygo to activate transcription. In addition to gaining a better understanding of how CtBP functions in the Wg/Wnt pathway, the tools developed in this study to uncouple CtBP activation and repression in Wg signalling can be utilized to explore the requirement of CtBP oligomerization in other contexts where CtBP has important biological roles.

Results

Monomeric CtBP activates Wg signalling in flies

CtBP is thought to exist in an equilibrium between monomeric (Kim *et al*, 2005; Zhao *et al*, 2009), homo-dimeric and possibly higher ordered homo-oligomeric complexes (Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Nardini *et al*, 2003; Shi *et al*, 2003; Thio *et al*, 2004; Kim *et al*, 2005; Mani-Telang *et al*, 2007; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009). While the native oligomeric state has mostly been determined for mammalian CtBP proteins, the entire dehydrogenase domain of fly CtBP is highly conserved (e.g. fly CtBP and hCtBP1 domains are 72% identical with 84% similarity). Nearly all of the residues making inter-molecular contact in the hCtBP1 homo-dimers are identical in fly CtBP (Kumar *et al*, 2002). This information was utilized to construct a fly CtBP protein that should not be able to dimerize, and thus remain monomeric.

There are several different isoforms of fly CtBP predicted to express proteins containing 383, 386, 476 and 479 residues (Poortinga *et al*, 1998; Nibu *et al*, 1998b; Sutrias-Grau and Arnosti, 2004). The short and long isoforms differ in their C-termini, downstream of the dehydrogenase domain. A minigene expressing a CtBP short isoform under the control of its endogenous regulatory elements can complement the *CtBP* mutant phenotype (Zhang and Arnosti, 2011). We have previously shown that both short and long isoforms can activate Wg/Arm-dependent transcription (Fang *et al*, 2006). Hence, a short isoform (383 aa) was used for all subsequent experiments in this report.

To generate a monomeric CtBP, four conserved residues, previously shown to be important for hCtBP1 self-association (Kumar *et al*, 2002), were mutated in fly CtBP. The resulting CtBP variant is referred to as $CtBP^{Mono}$. The C134Y and N138R substitutions should result in steric and electrostatic hindrance, hence preventing homo-dimerization and the R141A and R142A mutations should disrupt inter-molecular salt bridges and hydrogen bonds as predicted for hCtBP1 (Kumar *et al*, 2002). The normal equilibrium between monomers and dimers in wild-type CtBP ($CtBP^{WT}$) should be dramatically shifted to the monomeric state for $CtBP^{Mono}$ (Figure 1A).

To test if the mutations in $CtBP^{Mono}$ abolished its ability to self-associate, differentially tagged CtBP forms were co-transfected in the *Drosophila* hemocyte-derived cell line Kc167 (Kc) and assayed for binding using co-immunoprecipitation (co-IP). While $CtBP^{WT}$-Flag could co-IP $CtBP^{WT}$-HA, it was not able to pull-down $CtBP^{Mono}$-HA (Figure 1B). Mutations also

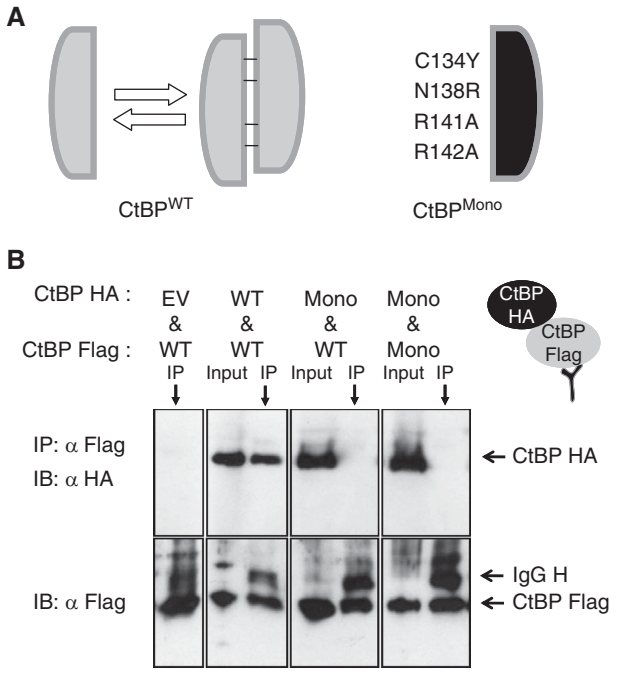

Figure 1 Conserved residues in the dimerization interface are required for self-association of CtBP. (**A**) Cartoon illustrating the equilibrium between monomers and dimers of $CtBP^{WT}$ and how four mutations in the dimerization interface in $CtBP^{Mono}$ shift the equilibrium to monomers. (**B**) $CtBP^{Mono}$ cannot associate with itself or $CtBP^{WT}$. A Flag-tagged version of $CtBP^{WT}$ can co-IP $CtBP^{WT}$-HA (top panel, lane 3). No signal was observed if $CtBP^{WT}$-HA was left out of the transfection (lane 1). In contrast to $CtBP^{WT}$, $CtBP^{Mono}$-HA did not co-IP with $CtBP^{WT}$-Flag (lane 5) or $CtBP^{Mono}$-Flag (lane 7). The Flag-tagged forms of $CtBP^{WT}$ or $CtBP^{Mono}$ were pulled down with a similar efficiency (lanes 1, 3, 5 and 7, bottom panel). Inputs (15% of total) for each co-IP are shown in lanes 2, 4 and 6.

disrupted the ability of $CtBP^{Mono}$ to homo-oligomerize as judged by this assay (Figure 1B). These results demonstrate that $CtBP^{Mono}$ cannot dimerize, resulting in a 'forced monomer' version of CtBP.

To test the role of dimerization in regulating Wg signalling, transgenic lines were constructed containing $CtBP^{WT}$ or $CtBP^{Mono}$ under the control of the Gal4/upstream activating sequence (UAS) inducible promoter. These CtBPs can then be expressed in any fly tissue for which a Gal4 driver line is available (Phelps and Brand, 1998). We previously reported that overexpression of CtBP, via nearby insertion of a P[GSV] element (Toba *et al*, 1999) could suppress a small eye phenotype caused by overstimulation of Wg signalling in the developing eye (Fang *et al*, 2006). Consistent with these results, several P[UAS-$CtBP^{WT}$] lines were able to suppress the small eye phenotype caused by GMR-Gal4-dependent expression of a stabilized form of Arm (Arm*) (Figure 2A and B). Thus, CtBP antagonizes Wg signalling downstream of Arm stabilization in this assay. In stark contrast, misexpression of $CtBP^{Mono}$ caused a significant enhancement of the *GMR-Gal4::UAS-arm** small eye phenotype (Figure 2A and C), suggesting that CtBP monomers promote Wg signalling in this context.

Wg signalling also has a significant role in defining the wing margin that originates from the dorsal/ventral (D/V) boundary of the wing imaginal disc. Antagonism of Wg signalling in this tissue leads to a loss of the wing margin, causing notches in the adult wing (Phillips and Whittle, 1993; Couso *et al*, 1994). To assay the role of $CtBP^{Mono}$ in Wg-directed wing margin formation, a sensitized genetic background was created by misexpression of Pygo at the anterior/posterior (A/P) boundary of the wing disc using *Patched-Gal4* (*Ptc-Gal4*) (Figure 2D–F). Although Pygo is known to positively regulate Wg signalling (Belenkaya *et al*, 2002; Kramps *et al*, 2002; Parker *et al*, 2002; Thompson *et al*, 2002), misexpression of Pygo antagonizes Wg signalling, possibly due to disruption of the stoichiometry of a protein complex (Parker *et al*, 2002).

In the *Ptc-Gal4::UAS-Pygo* background employed in this assay, over 80% of the adult wings displayed notches. These notches were categorized into two groups based on their size. Small notches had loss of wing margin only between the L3 and L4 veins (see Figure 2E). Big notches extended beyond these veins (see Figure 2F). Co-expression of $CtBP^{Mono}$ significantly suppressed the loss of wing margin caused by Pygo, with a dramatic reduction in the frequency of big notches (Figure 2G). These data provide another line of evidence supporting a positive role for $CtBP^{Mono}$ in the regulation of Wg signalling.

To further test the role of $CtBP^{Mono}$ in Wg signalling, expression of a Distalless enhancer trap line (*Dll-lacZ*) was monitored. In larval third instar wing imaginal discs, *Dll-lacZ* is activated by Wg in a broad domain centred on the D/V boundary of the presumptive wing blade (Zecca *et al*, 1996; Neumann and Cohen, 1997) (Figure 3A). Transgenic flies carrying *UAS-*$CtBP^{WT}$ or *UAS-*$CtBP^{Mono}$ transgenes were crossed to a *Engrailed-Gal4 (En-Gal4)* driver, leading to expression of transgenes in the posterior half of the disc (Figure 3E and H). Lines expressing $CtBP^{WT}$ and $CtBP^{Mono}$ at similar levels resulted in an enhancement in the *Dll-lacZ* expression (Figure 3D, F, G and I; Supplementary Figure S1). These results provide additional support for positive regulation of the Wg pathway by $CtBP^{Mono}$.

CtBP monomers promote activation of Wg targets in Kc cells and CtBP acts downstream of Pygo in activating transcription

We have previously shown that the expression of the genes *CG6234* and *naked cuticle (nkd)* is activated by Wg signalling in Kc cells (Fang *et al*, 2006). In the absence of signalling, CtBP and TCF act in parallel to repress *nkd* expression, but CtBP is not required for activation of *nkd* expression by Wg signalling (Fang *et al*, 2006). In contrast, CtBP repression of *CG6234* in the absence of signalling is minimal, but CtBP is required for maximal activation of *CG6234* upon Wg stimulation (Fang *et al*, 2006).

To test whether the positive regulation of *CG6234* by CtBP is occurring at the transcriptional level, a reporter gene containing a minimal WRE from this target gene was examined. Figure 4A shows the location of a minimal WRE (539 bp) derived from a previously reported 2.2 kb WRE (Fang *et al*, 2006; see Materials and methods), which is comparatively more responsive to Wg signalling (data not shown).

The *CG6234* WRE reporter was highly activated by expression of Arm* in a TCF-dependent manner (Figure 4B). RNAi-mediated depletion of *CtBP* also caused a dramatic reduction in activation of the *CG6234* WRE reporter (Figure 4B). Consistent with the data for *CG6234* transcripts (Fang *et al*, 2006), there was still residual activation of the *CG6234* WRE

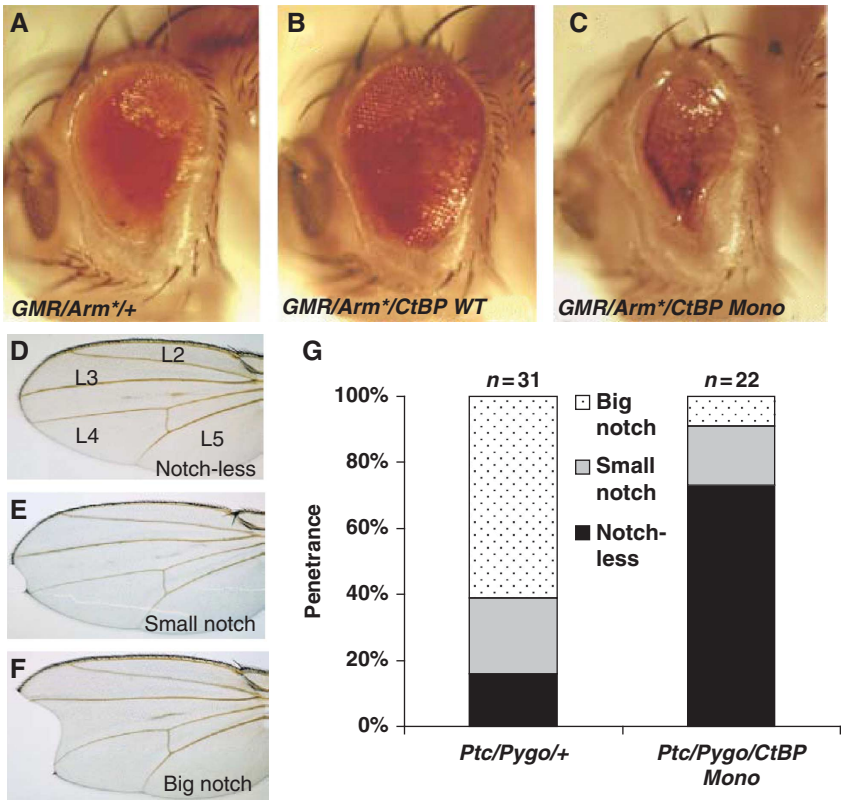

Figure 2 CtBP^{Mono} positively regulates Wg signalling in fly tissues. (**A**–**C**) Adult eyes of *GMR-Gal4::UAS-Arm** flies containing no transgene (**A**), *UAS-CtBP^{WT}* (**B**) or *UAS-CtBP^{Mono}* (**C**). The reduction in the fly eye size caused by Arm* expression is suppressed by co-expression CtBP^{WT} and is enhanced by CtBP^{Mono}. (**D**–**F**) Representative adult wings from *Ptc-Gal4::UAS-Pygo* flies that either lack a notch, or contain a small notch (between the L3 and L4 vein) or big notch (between the L2 and L5 vein) due to antagonism of Wg signalling. (**G**) The effect of CtBP^{Mono} on the frequency of the *Ptc-Gal4::UAS-Pygo*-dependent notches. CtBP^{Mono} causes a marked reduction in the frequency and size of the wing notches.

reporter by Arm* in *CtBP* RNAi-treated cells (Figure 4B). These data suggest that CtBP acts in parallel with other co-activators, which are recruited by Arm for activation of *CG6234* WRE reporter.

To test the role of CtBP dimerization in promoting Wg-mediated transcriptional activation of the *CG6234* WRE reporter, a *CtBP* gene replacement strategy was employed. Endogenous *CtBP* was depleted using dsRNA corresponding to the 5′UTR of *CtBP*. These cells were then transfected with the reporter, plus transgenes expressing CtBP^{WT} or CtBP^{Mono}. These *CtBP* transgenes contained a heterologous 5′ UTR, so they were not targeted by the *CtBP* RNAi. Activation of the *CG6234* WRE reporter by Wg-conditioned media was then assayed. Transfection of CtBP^{Mono} rescued the *CtBP* RNAi defect to a similar level as seen with CtBP^{WT} transfection (Figure 4C; compare the second and third groups). A similar rescue of *CG6234* WRE reporter activation by CtBP^{Mono} was observed when the Wg pathway was stimulated by expression of Arm* (Figure 4D). CtBP^{Mono} was expressed at similar levels as CtBP^{WT} in these experiments (Figure 4D and data not shown). These results demonstrate that CtBP^{Mono} is capable of substituting for endogenous CtBP to promote activation of the *CG6234* WRE reporter.

CtBP has previously been shown to be required for activation of Gal4–Arm*-dependent activation of a *UAS-luc* reporter (Fang *et al*, 2006). Both CtBP^{WT} and CtBP^{Mono} had no effect on *UAS-luc* when co-expressed with Gal4DBD (Gal4 DNA-binding domain). However, both CtBP forms dramatically enhanced the ability of Gal4–Arm* to activate *UAS-luc* (Figure 4E). Taken together with the data from Figure 4D, these results indicate that like CtBP^{WT}, CtBP^{Mono} is functioning downstream of Arm to activate Wg transcriptional targets.

Arm contains at least two domains that contribute to activation of gene expression, a N-terminal and a C-terminal transactivation domain (Hecht *et al*, 1999; Stadeli and Basler, 2005; Fang *et al*, 2006). CtBP is recruited by the N-terminal domain of Arm for transcriptional activation (Fang *et al*, 2006). The N-terminal domain of Arm binds to Lgs, which serves as an adaptor to recruit Pygo to the TCF–Arm complex (Kramps *et al*, 2002). In order to explore a possible connection between CtBP and Pygo in activating transcription, we tested the ability of a Gal4–Pygo fusion protein to activate the *UAS-luc* reporter when endogenous CtBP was depleted. The activation of *UAS-luc* reporter by Gal4–Pygo was markedly reduced in cells treated with CtBP RNAi (Figure 4F). Gal4–Pygo has previously been shown to activate transcription independently of Arm and Lgs (Stadeli and Basler, 2005), suggesting that CtBP acts downstream of Pygo to activate Wg target gene expression.

CtBP dimerization is required for its antagonistic role in Wg signalling in Kc cells

We have previously identified three *WREs* in the *nkd* locus, two upstream of the *nkd* transcriptional start site (*nkd-UpE1* and *nkd-UpE2*) and one in the first intron of *nkd* (*nkd-IntE*) (Chang *et al*, 2008). While reporters for all three WREs were

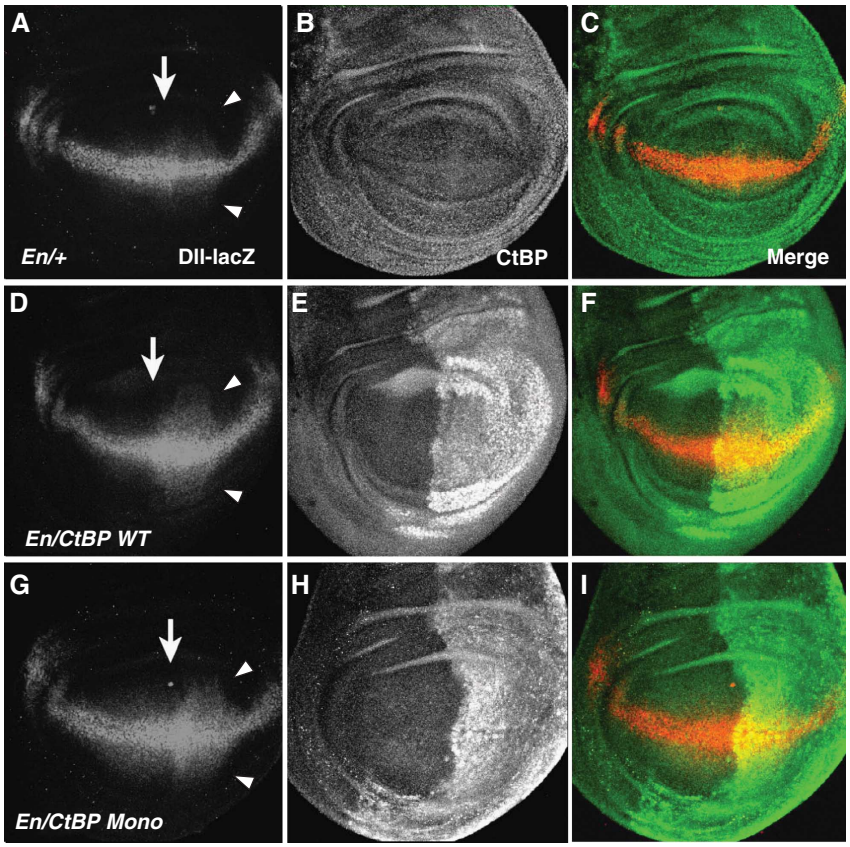

Figure 3 CtBP^{Mono} positively regulates the Wg reporter *Dll-lacZ in vivo*. (**A**–**I**) Confocal images of third instar wing imaginal discs stained for *Dll-lacZ* (**A**, **D**, **G**) and CtBP (**B**, **E**, **H**) expression from animals containing *En-Gal4* with no transgene (**A**–**C**), *UAS-CtBP^{WT}* (**D**–**F**) or *UAS-CtBP^{Mono}* (**G**–**I**). *En-Gal4* drives CtBP^{WT} or CtBP^{Mono} expression at similar levels in the posterior compartment of the disc (**B**, **E** and **H**; white arrows in **A**, **D** and **G** mark the A/P boundary). Expression of either CtBP^{WT} or CtBP^{Mono} enhances the expression of *Dll-lacZ* (see white arrowheads).

derepressed upon depletion of *CtBP*, the *nkd-UpE1* reporter consistently exhibited the largest response (Supplementary Figure S2). In addition, *TCF* knockdown also caused derepression of *nkd-UpE1*, while having no effect on *nkd-UpE2* or *nkd-IntE* (Supplementary Figure S2). Therefore, *nkd-UpE1* (Figure 5A) was used for all subsequent experiments to assay the role of CtBP oligomers in regulating this *nkd* WRE. Similar to *nkd* mRNA (Fang *et al*, 2006), simultaneous knockdown of *CtBP* and *TCF* resulted in a far greater derepression of *nkd-UpE1* reporter activity than with either factor alone (Figure 5B). TCF has already been shown to be enriched at the *UpE1* WRE (Chang *et al*, 2008). As judged by chromatin IP (ChIP), CtBP was also enriched at the *nkd-UpE1* (Supplementary Figure S3), supporting a direct role for CtBP in repression of this WRE.

To test if dimerization of CtBP was required for inhibition of Wg targets in the absence of signalling, the ability of CtBP^{Mono} to repress the *nkd-UpE1* reporter was assayed. This was done using a similar gene replacement strategy as described in Figure 4. As expected, transiently expressed CtBP^{WT} was able to repress the *nkd-UpE1* in the absence of signalling. Strikingly, CtBP^{Mono} was unable to perform this function (Figure 5C). CtBP^{Mono} sometimes caused greater derepression of *nkd-UpE1* than the control (Figure 5C), possibly due to a weak dominant negative effect on CtBP repressive activity, but this effect was not always observed (see Figure 6D). Mutations that abolish self-association of CtBP, while having no affect on its ability to promote Wg signalling, severely disrupt its ability to repress Wg target gene expression in the absence of signalling.

The approach described above is similar to that used in several other studies to provide evidence that CtBP dimerization is required for transcriptional repression (Kumar *et al*, 2002; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009), that is, correlating loss of dimerization with loss of CtBP activity. However, this approach cannot rule out that the mutations disrupting homo-dimerization also affect other aspects of CtBP function. To provide a more convincing demonstration of the importance of CtBP self-association in antagonizing Wg signalling, monomeric versions of CtBP were created that cannot homo-dimerize, yet possess the ability to hetero-dimerize with each other. If dimerization is essential for repression by CtBP, then the monomeric forms should not be able to repress Wg targets but co-expression of these complementary monomeric forms should reconstitute dimerization and hence the repressive function of CtBP.

The strategy for engineering complementary monomeric forms of CtBP required identifying the salt bridges in the CtBP dimer and then switching the positions of the acidic and basic residues forming the salt bridge. Such inter-molecular salt bridge swaps have been previously used to show interaction or self-association of various proteins (Xiao *et al*, 1999; Watt *et al*, 2001; Venkatachalan and Czajkowski, 2008). Using the structural information of the highly conserved hCtBP1, two

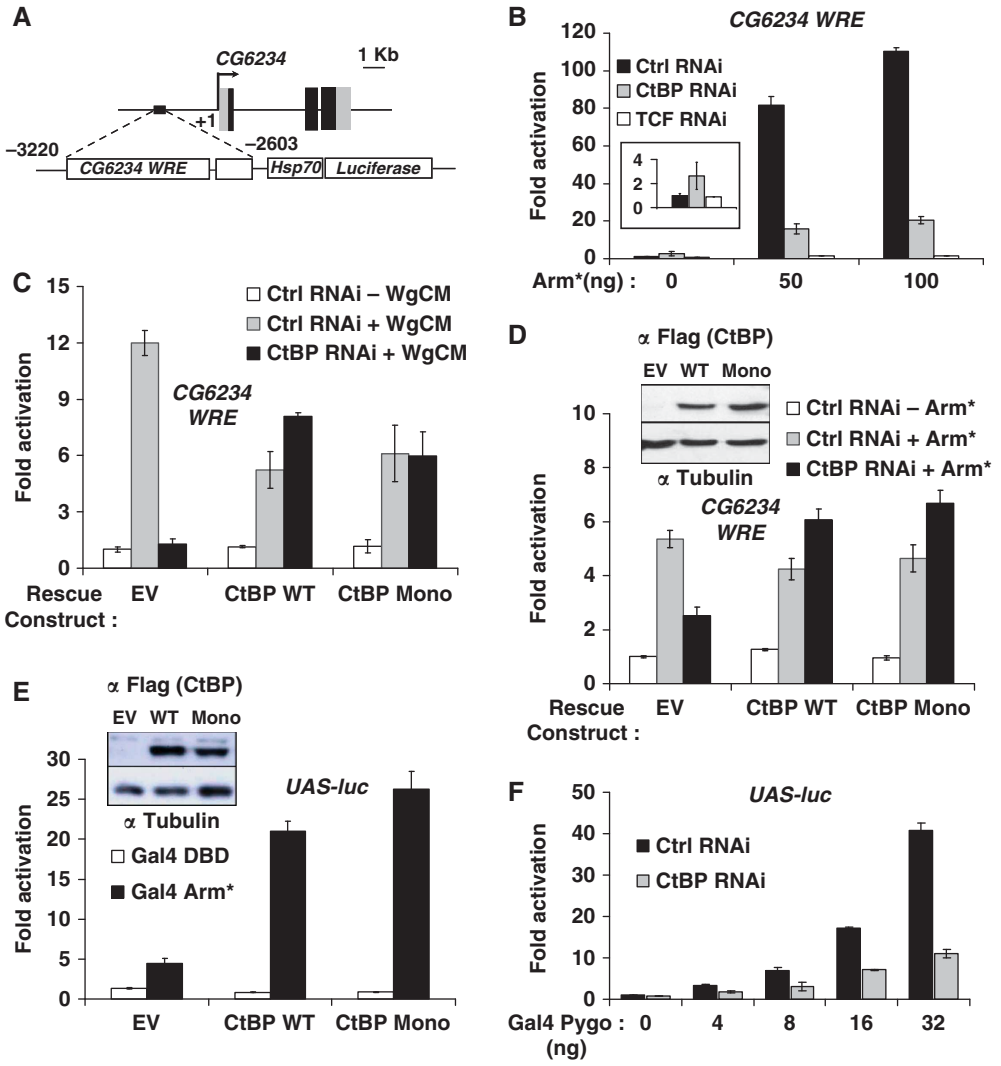

Figure 4 CtBP monomers activate Wg targets in Kc cells and CtBP is required for Pygo transactivation activity. (**A**) Schematic showing the location of the WRE in relation to the *CG6234* transcription start site and the WRE inserted upstream of a minimal *Hsp70* core promoter/luciferase gene cassette, constituting the *CG6234* WRE reporter. (**B**) Dose-dependent activation of *CG6234* WRE reporter by Arm* in Kc cells is attenuated when endogenous *CtBP* or *TCF* is depleted by RNAi to either ORFs in Kc cells. Note that some activation of the *CG6234* WRE reporter still occurs when CtBP is depleted. A maximum of 2.6-fold derepression was noted upon *CtBP* knockdown in the absence of Arm* (inset). (**C**) $CtBP^{Mono}$ is able to rescue the Wg-CM-mediated stimulation of the *CG6234* WRE reporter when endogenous *CtBP* was depleted. (**D**) $CtBP^{Mono}$ rescued *CG6234* WRE activation by Arm* to a similar level as $CtBP^{WT}$. Both CtBP proteins were expressed at similar levels as judged by immunoblots (inset). (**E**) $CtBP^{Mono}$ was able to enhance the Gal4–Arm*-mediated activation of *UAS-luc* as efficiently as $CtBP^{WT}$ when expressed at similar levels (inset). (**F**) Dose-dependent activation of the *UAS-luc* reporter by Gal4–Pygo is significantly reduced upon knockdown of endogenous *CtBP*. Each bar represents a mean of luciferase values from cultures transfected in duplicate (± s.e.), except for panels (**E**, **F**), which were in triplicate. Each result is representative of at least three independent experiments, except for panel (**F**), which was performed twice.

salt bridges (E126–R173 and E127–R171) formed by conserved residues were targeted for a swap. $CtBP^{Basic}$ contains E126R and E127R substitutions, while $CtBP^{Acidic}$ has R173E and R171E alterations. When expressed on their own, $CtBP^{Basic}$ and $CtBP^{Acidic}$ should be monomeric but have the ability to form $CtBP^{Basic}$/$CtBP^{Acidic}$ hetero-dimers when co-expressed (Figure 6A).

As expected, co-expression of $CtBP^{Basic}$ and $CtBP^{Acidic}$ led to association of these molecules at levels similar to those seen with $CtBP^{WT}$ (Supplementary Figure S4; Figure 6B, lane 2). Co-expression of differentially tagged versions of $CtBP^{Acidic}$ did not result in an appreciable co-IP (Figure 6B, lane 1). Assaying self-association of $CtBP^{Basic}$ was complicated by the fact that the V5-tagged version of this protein was somewhat unstable when expressed with a Flag-tagged $CtBP^{Basic}$ (Figure 6C, lane 1). Stability was greatly increased by co-expression with $CtBP^{Acidic}$ (Figure 6C, lane 2). Although V5-tagged $CtBP^{Acidic}$ was more readily expressed, it also appeared to be more stable in the presence of the complementary $CtBP^{Basic}$ (Figure 6C, lanes 3 and 4). In contrast to the V5-tagged proteins, the Flag-tagged versions were relatively stable when expressed under all conditions (Figure 6C). Taken together, these data demonstrate that the $CtBP^{Acidic}$ and $CtBP^{Basic}$ mutants function as predicted, being unable to homo-dimerize but capable of efficient hetero-dimerization.

When tested for their ability to rescue the derepression of the *nkd-UpE1* reporter in cells depleted of endogenous CtBP, neither $CtBP^{Acidic}$ nor $CtBP^{Basic}$ were able to provide significant repressive activity, similar to the original $CtBP^{Mono}$ mutant (Figure 6D). Remarkably, co-expression of $CtBP^{Basic}$

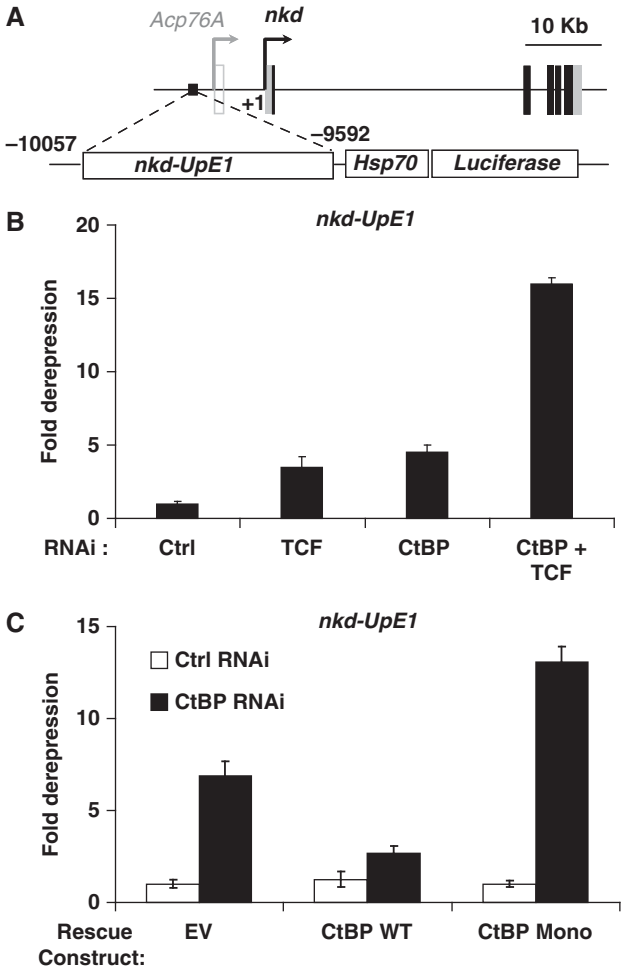

Figure 5 CtBP monomers are unable to repress the *nkd-UPE1* WRE in the absence of signalling in Kc cells. (**A**) Schematic showing the location to the *UpE1* WRE in relation to the *nkd* transcriptional start site and outlining the construction of the *nkd-UpE1* luciferase reporter. (**B**) The *UpE1* reporter in Kc cells shows a derepression when endogenous *CtBP* or *TCF* is depleted by RNAi that is greatly enhanced when *CtBP* and *TCF* are knocked down simultaneously. (**C**) Exogenous $CtBP^{Mono}$ cannot rescue the repression of *nkd-UpE1* reporter when endogenous *CtBP* is knocked down, but $CtBP^{WT}$ is able to silence *UpE1* when expressed exogenously in Kc cells. For all experiments, each bar represents a mean of luciferase values from cultures transfected in triplicate (± s.e.). All experiments are representative of at least three independent experiments.

and $CtBP^{Acidic}$ restored the inhibition of *nkd-UPE1* in the absence of signalling, to a similar extent as observed with $CtBP^{WT}$. These data provide compelling evidence that self-association is required for the function of CtBP as a co-repressor of a Wg transcriptional target.

CtBP dimerization is required for its ability to inhibit wing margin formation

To extend the findings summarized in Figures 5 and 6 to fly tissues, the ability of $CtBP^{WT}$ and $CtBP^{Mono}$ to affect development of the wing was examined. Expression of $CtBP^{WT}$ at the A/P boundary of the wing disc using the *Decapentaplegic-Gal4* (*Dpp-Gal4*) driver gave rise to a high frequency of wing notches (Table I). Expression of CtBP had no effect on the expression of Wg (Supplementary Figure S5). This phenotype is consistent with a reduction in Wg signalling at the presumptive wing margin. In contrast, expression of $CtBP^{Mono}$ never resulted in wing notching (Table I). These data provide further support for a model where CtBP self-association is required for the ability of CtBP to antagonize events downstream of Wg expression in the wing primordium.

To confirm that CtBP dimerization was required for inhibition of wing margin formation, $CtBP^{Basic}$ and $CtBP^{Acidic}$ transgenes were also tested for a wing phenotype when misexpressed using *Dpp-Gal4* (Table I). Flies containing two UAS transgenes each were generated in the following combinations: Acidic/Acidic, Basic/Basic or Acidic/Basic. While expression of both complementary combinations ($Acidic^1$/$Basic^1$ or $Acidic^2$/$Basic^2$) resulted in significant wing notching, $Acidic^1$/$Acidic^2$ or $Basic^1$/$Basic^2$ combinations did not (Table I). Immunostaining with CtBP antisera was performed to ensure that comparisons were made with CtBP variant proteins expressed at similar levels (Supplementary Figure S5). The CtBP $Acidic^1$/$Basic^1$ and $Acidic^1$/$Acidic^2$ backgrounds were expressed at similar levels, while the $Basic^1$/$Basic^2$ and $Acidic^2$/$Basic^2$ combinations were expressed at slightly lower levels (Supplementary Figure S5). As with $CtBP^{WT}$ and $CtBP^{Mono}$, Wg expression at the presumptive margin was not affected by any of the Acidic/Basic combinations (Supplementary Figure S5). These results indicate that dimerization of CtBP is required for antagonism of Wg signalling during wing margin formation.

A monomeric pool of CtBP in Kc cells

To assess the distribution of the monomeric and oligomeric pool of CtBP in cultured Kc cells, the association of V5-tagged $CtBP^{WT}$ (WT V5) and Flag-tagged $CtBP^{WT}$ (WT Flag) was examined. Excess WT Flag was expressed, in order to drive most of the WT V5 into a heteromeric complex (Figure 7A, lanes 9–12). As expected, WT V5 was found to associate with WT Flag (Figure 7A, lanes 5–8). In addition, there was a considerable amount of WT V5 present in the FLAG immunodepleted supernatants, even when a 10-fold higher level of WT Flag was expressed (Figure 7A, lane 4). This suggests the existence of a substantial monomeric pool of CtBP.

In order to exclude the possibility that the non-precipitated WT V5 CtBP was in a homo-oligomeric state, a similar immunodepletion was performed using V5-tagged $CtBP^{Acidic}$ (Acidic V5) and Flag-tagged version of $CtBP^{Basic}$ (Basic Flag) (Figure 7B). These CtBP mutants are unable to form homo-oligomers (Figure 6B). An increasing dose of Basic Flag was expressed (Figure 7B, lanes 9–12), and found to associate with Acidic V5 (Figure 7B, lanes 5–8). As was found with WT CtBPs, there was a substantial amount of Acidic V5 in the immunodepleted supernatants, even when 10-fold higher Basic Flag was expressed (Figure 7B, lane 4). These data support the view that a significant pool of CtBP is present as monomers in Kc cells.

Discussion

The oligomeric state of CtBP determines its effect on Wg signalling

CtBP is well known for its role as a co-repressor for many transcription factors (Turner and Crossley, 2001; Chinnadurai, 2007; Kuppuswamy *et al*, 2008). It is also known to antagonize Wnt/β-cat signalling, possibly by binding to some TCFs (Brannon *et al*, 1999; Valenta *et al*, 2003) or by acting with

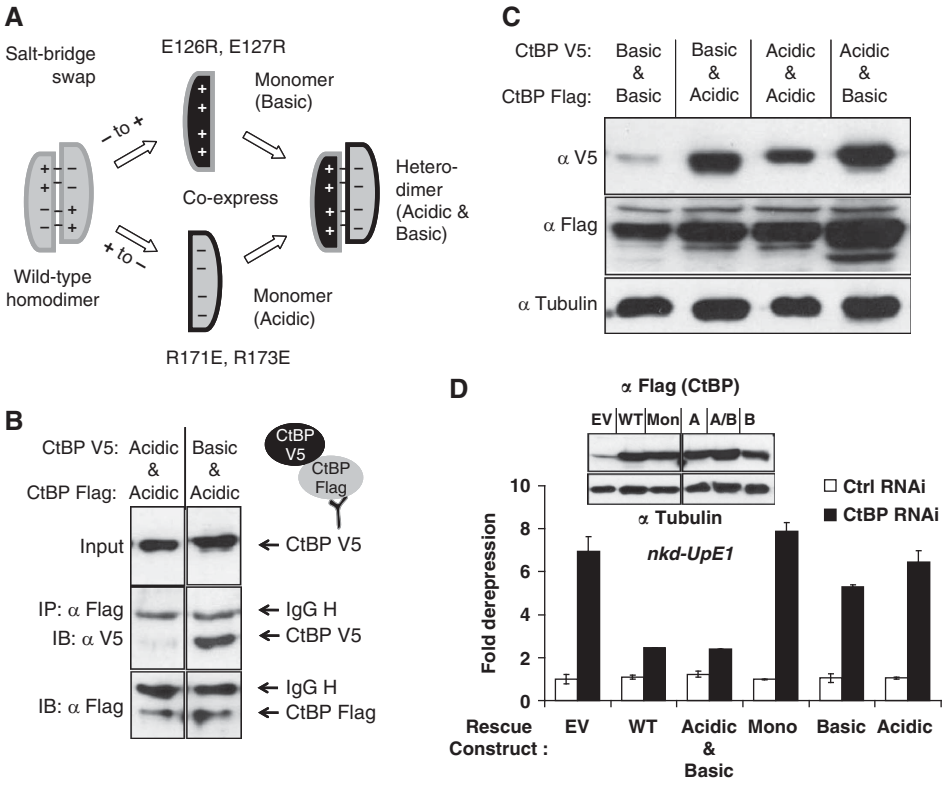

Figure 6 CtBP dimerization is required for silencing the *nkd-UpE1* reporter in the absence of signalling. (**A**) Cartoon outlining the rationale for creating two monomeric CtBP forms ($CtBP^{Acidic}$ and $CtBP^{Basic}$) that can hetero-dimerize. (**B**) $CtBP^{Acidic}$ cannot co-IP itself but can pull down $CtBP^{Basic}$. When expressed in Kc cells, a Flag-tagged form of $CtBP^{Acidic}$ was unable to pull down a V5-tagged form of $CtBP^{Acidic}$ (lane 1, middle panel) but co-IP was observed with a V5-tagged form of $CtBP^{Basic}$ (lane2, middle panel). Flag-tagged forms of $CtBP^{Acidic}$ were pulled down with a similar efficiency (bottom panel). Input (15% of total) are shown in the top panels. (**C**) Immunoblots of Kc cells expressing the indicated CtBP mutants with antibodies against the Flag or V5 epitopes showing that V5-tagged $CtBP^{Basic}$ is stable only when co-expressed with $CtBP^{Acidic}$. V5 and Flag-tagged versions of $CtBP^{Acidic}$ were also expressed at higher levels when co-expressed with a complementary $CtBP^{Basic}$. The same amounts of CtBP expression vector were transfected in all lanes. (**D**) Co-expression of $CtBP^{Acidic}$ and $CtBP^{Basic}$ reconstitutes the ability of CtBP to repress the *nkd-UpE1* reporter. Kc cells were depleted of endogenous CtBP by a dsRNA corresponding to the 5′ UTR. This resulted in a seven-fold derepression in *nkd-UpE1* reporter activity, which was largely rescued by expression of $CtBP^{WT}$. However, $CtBP^{Mono}$, $CtBP^{Basic}$ and $CtBP^{Acidic}$ are unable to repress *the nkd-UpE1* reporter, and co-expression of $CtBP^{Acidic}$ with $CtBP^{Basic}$ resulted in a similar degree of repression as $CtBP^{WT}$. All the Flag-tagged CtBP mutants used were expressed at similar levels (inset). Each bar represents a mean of luciferase values from cultures transfected in duplicate (±s.e.). For each experiment, the result shown here is representative of at least three independent experiments.

Table I $CtBP^{Acidic/Basic}$ antagonizes Wg signalling during wing development

Dpp-Gal4 > UAS-CtBP (°C)	Notches (%)
WT (27)	50.2
$Acidic^1/Basic^1$ (27)	43.6
$Acidic^2/Basic^2$ (27)	36.0
$Acidic^1/Acidic^2$ (27)	0.0
$Basic^1/Basic^2$ (29)	0.0
Mono (29)	0.0

Percentage of notched wings ($n > 100$ for each genetic background) upon co-expression of CtBP trangenes using *Dpp-Gal4*. Flies were reared at 27 or 29°C to equalize the level of CtBP expression. Two versions of $CtBP^{Basic}$ and $CtBP^{Acidic}$ (1 and 2) were used, so that the transgene copy number was equal when comparing Acidic/Acidic, Basic/Basic and Acidic/Basic wings.

APC to divert β-catenin away from TCF (Hamada and Bienz, 2004; Sierra *et al*, 2006). In *Drosophila* Kc cells, we have previously shown that CtBP works in parallel with TCF to repress expression of the Wg target *nkd* in the absence of signalling (Fang *et al*, 2006). In addition, we found that CtBP was required for activation of several Wg targets in cultured cells and fly tissues (Fang *et al*, 2006). Our data indicate that CtBP can both repress and activate the Wg pathway in a gene-specific manner.

In this report, we provide a dramatic example of this differential regulation of Wg-mediated transcription by CtBP using *WRE* reporter constructs. While CtBP is required for silencing the *nkd-UpE1* reporter in the absence of signalling (Figures 5 and 6), depletion of CtBP results in a significant reduction of the *CG6234* WRE reporter activation upon Wg stimulation (Figure 4). Since both these *WREs* are directly activated by TCF–Arm (Fang *et al*, 2006; Chang *et al*, 2008), these results indicate that additional sequence information must exist in these elements that influence CtBP's relationship with TCF and Arm. Our findings that CtBP is required for activation of a simple *UAS-luc* reporter by Gal4–Arm* and Gal4–Pygo fusion proteins (Fang *et al*, 2006; Figure 4E and F), suggests that activation by CtBP might be the default state for the Wg pathway.

How can CtBP both promote and repress transcription of Wg targets? Our data demonstrate that the quaternary state of the CtBP protein determines its role as an activator and repressor. CtBP mutants that cannot homo-dimerize are unable to repress *nkd-UpE1* expression (Figures 5C and 6D) or

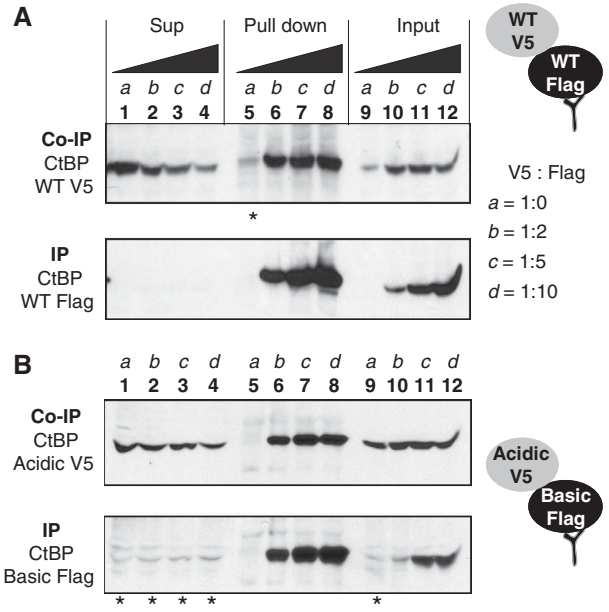

Figure 7 A significant amount of CtBP is present in the monomeric pool in Kc cells. Immunoblot showing the anti-Flag immunodepleted supernatant (Sup; lanes 1–4), immunoprecipitate (pull down; lanes 5–8) and expression (Input; lanes 9–12) for (**A**) CtBP WT V5 and CtBP WT Flag and (**B**) $CtBP^{Acidic}$ V5 and $CtBP^{Basic}$ Flag. CtBP V5:CtBP Flag were co-expressed in ratios of 1:0(a), 1:2(b), 1:5(c) and 1:10(d) and then lysates were immunoprecipitated with anti-Flag antibody and pull-down CtBP Flag and CtBP V5 determined by western blot. There is a significant amount of CtBP V5 present in the supernatant (lane 4, top panels (**A**, **B**)), even in lysates where a large excess of CtBP Flag was immunoprecipitated (lane 4, bottom panels (**A**, **B**)). Lanes containing a cross-reacting protein running at a similar molecular weight as CtBP are indicated with '*'.

inhibit wing margin formation (Table I). However, co-expression of complimentary monomeric CtBP mutants that can hetero-dimerize restores CtBP repression activity in both these readouts (Figure 6D and Table I). This provides a compelling argument that self-association of CtBP is required for its ability to antagonize Wg transcriptional targets.

In contrast to targets where CtBP inhibits Wg signalling, monomeric CtBP can rescue the loss of Wg activation of the *CG6234* WRE reporter in Kc cells depleted of endogenous CtBP (Figure 4C and D). In addition, CtBP monomers enhance an Arm-induced small eye phenotype (Figure 2C), can rescue a weak loss of Wg signalling defect in the fly wing (Figure 2G) and activate the Wg target *Dll-lacZ* (Figure 3; Supplementary Figure S1). Taken together, our data strongly support a model where CtBP monomers activate and CtBP dimers repress the Wg pathway in a gene-specific manner.

Normal or monomeric CtBP promote expression of *UAS-luc*, *CG6234-luc* and *Dll-lacZ* (Figures 3 and 4). In contrast, wild-type or hetero-dimeric CtBP represses the *nkd-UpE1* reporter while monomeric CtBP cannot (Figures 5 and 6). The situation for CtBP's action on eye size in a GMR-Arm* genetic background is more complicated, with WT CtBP suppressing the reduction in eye size (Figure 2B) while monomeric CtBP enhances the GMR-Arm*-induced small eye phenotype (Figure 2C). The same is true for the wing margin, where monomeric CtBP can rescue the loss of wing margin caused by a reduction in Wg signalling (Figure 2D–G), while WT CtBP promotes wing notching (Table I). We suspect that unlike other Wg readouts, where either the activation or repressing function of CtBP is dominant, in the GMR-Arm* eye and at the wing margin, both activities are prevalent, with the repressive function winning out in oligomeric CtBP expression and the activation function with monomeric CtBP.

Mechanism of CtBP action on Wg signalling

Why is dimerization required for repression by CtBP? CtBPs have been shown to bind to many transcriptional repressors and some interactions require the dimerization of CtBP (Turner and Crossley, 2001; Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Chinnadurai, 2007; Kuppuswamy *et al*, 2008). Although homodimerization is dispensable for interaction of CtBP with some factors, it is clearly required for the function of CtBP as a potent co-repressor in complex with those factors (Kuppuswamy *et al*, 2008; Zhao *et al*, 2009). CtBPs have also been shown to associate with several chromatin-modifying enzymes, which have crucial roles in transcriptional repression (Shi *et al*, 2003; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009). A recent report provides good evidence that in the case of repression of *E-Cadherin* by the repressor ZEB, human CtBP2 dimers act as adaptors between ZEB and histone deacetylase 2 (HDAC2) (Zhao *et al*, 2009). Binding of ZEB and HDAC2 to CtBP monomers was mutually exclusive, necessitating the need for CtBP dimerization to form the ZEB–CtBP–HDAC2 complex (Zhao *et al*, 2009). It is possible that this mechanism is also operating in the silencing of *nkd* expression in Kc cells, though further work is required to identify the binding partners of CtBP in this system.

In the case of activation, we have previously shown that CtBP functionally interacts with the N-terminal transactivation domain of Arm (Fang *et al*, 2006). This domain of Arm is bound by Lgs, which is in a complex with Pygo to promote transcriptional activation (Kramps *et al*, 2002; Stadeli and Basler, 2005; Li *et al*, 2007). Here, we extend our understanding of the mode of action of CtBP in activating Wg targets by demonstrating that CtBP substantially contributes to transcriptional activation by a Gal4–Pygo fusion protein (Figure 4F). Since Gal4–Pygo acts downstream of Arm and Lgs in activating transcription (Stadeli and Basler, 2005), our results indicate that CtBP acts downstream of Pygo.

The requirement of CtBP for Pygo activity is interesting in light of the genetic interaction data between pygo and monomeric CtBP in the developing wing. Overexpression of *pygo* causes notches in the wing margin, which is partially rescued by expression of monomeric CtBP (Figure 2D–G). Expression of *pygo* blocks Wg signalling in several contexts (Parker *et al*, 2002), presumably by disrupting the stoichiometry of a Lgs–Pygo–unknown factor(s) complex (i.e. shifting the equilibrium to Lgs–Pygo and Pygo–unknown factor hetero-dimers). The data in Figure 2D–G are consistent with a model where expression of monomeric CtBP shifts the equilibrium back to a trimeric, Lgs–Pygo–CtBP complex, which can promote activation of Wg targets in the wing margin.

The Lgs–Pygo complex is generally required for Wg signalling throughout fly development (Belenkaya *et al*, 2002; Parker *et al*, 2002; Thompson *et al*, 2002). In Kc cells, Pygo is required for activation of both *CG6234* and *nkd* by Wg signalling (Parker and Cadigan, unpublished data). This suggests that there are other factors involved to explain why only a subset of Wg targets require CtBP for activation. In addition, Pygo has also been demonstrated to regulate

some Wg targets in the absence of signalling, suggesting a possible link with CtBP in this context (de la Roche and Bienz, 2007; Mieszczanek *et al*, 2008).

Regulation of the oligomeric state of CtBP

Several studies have demonstrated that CtBP can self-associate (Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Nardini *et al*, 2003; Shi *et al*, 2003; Thio *et al*, 2004; Kim *et al*, 2005; Mani-Telang *et al*, 2007; Kuppuswamy *et al*, 2008; Zhao *et al*, 2009). However, the presence of a monomeric pool has not been as extensively demonstrated (Kim *et al*, 2005; Zhao *et al*, 2009). To assess the distribution of monomers versus oligomers of CtBP, differentially tagged forms of CtBP were expressed. Even a 10-fold higher dose of CtBP-Flag could not completely co-immunoprecipitate CtBP-V5 (Figure 7A), indicating that a significant pool of CtBP-V5 is present as monomers or homo-dimers. Because similar results were obtained using $CtBP^{Basic}$ and $CtBP^{Acidic}$-tagged forms (Figure 7B), which prevent homo-oligomerization, our data support a model where monomeric and homo-oligomeric pools of CtBP are present in Kc cells.

CtBPs are highly homologous to NAD^+/NADH-dependent dehydrogenases and can bind NADH with high affinity (Fjeld *et al*, 2003). However, the role of co-factor based differential regulation of CtBP oligomerization is controversial. An increase in the NAD^+/NADH levels stimulates dimerization of mammalian CtBPs (Kumar *et al*, 2002; Balasubramanian *et al*, 2003; Thio *et al*, 2004; Kim *et al*, 2005). Mutations in the NAD^+/NADH-binding site of CtBPs abolish or reduce oligomerization (Kumar *et al*, 2002; Thio *et al*, 2004; Kuppuswamy *et al*, 2008; Nardini *et al*, 2009), although NAD^+ binding is not always essential for dimerization of CtBP (Mani-Telang *et al*, 2007). In the case of the short isoform of fly CtBP, mutations in the NAD-binding cleft (G181V, G183V) make the protein highly unstable in Kc cells (Bhambhani and Cadigan, unpublished data). A more stable NAD cleft mutant (D204N) has nuclear localization defects and is unable to rescue CtBP mutant flies (Zhang and Arnosti, 2011). This precludes any functional studies to test if the NAD^+/NADH ratio might affect its role in regulating Wg targets.

Does stimulation by Wg influence the oligomeric state of CtBP? In the absence of Wnt signalling, TCF acts with many other co-repressors to silence target gene expression. This repression is then counteracted by Arm/β-cat binding to TCF (Parker *et al*, 2007; Mosimann *et al*, 2009). Given the fact that CtBP dimers repress some Wg targets and CtBP monomers promote the Wg-dependent activation of some targets, it is tempting to speculate that Wg signalling causes a conversion of CtBP dimers to monomers. However, we have been unable to detect any difference in CtBP self-association with or without pathway activation in our co-IP assay (Supplementary Figure S6). Perhaps a more sensitive assay is required to detect changes in the oligomeric state upon Wg signalling.

An alternative to the Wg pathway influencing the oligomeric state of CtBP is a model where a pool of CtBP monomers and dimers exists in the cell in equilibrium (Figure 7). These pools might be differentially recruited to Wg targets by selective protein–protein interactions. In the case of CtBP monomers, this recruitment to WRE is predicted to require Arm. Although Wg signalling does not appear to influence the overall CtBP concentration in fly tissues (Fang *et al*, 2006), protein–protein interactions may cause changes in the monomer–dimer ratio on the WRE chromatin.

The role of CtBP oligomerization in other systems

The reagents and methodology described in this report can be applied to other systems where CtBP has important roles in regulating gene expression. For example, loss of *CtBP1* and *CtBP2* in the mouse results in loss of posterior structures in the embryo, a phenotype that has many similarities to Wnt3a mutants (Hildebrand and Soriano, 2002). This suggests that mammalian CtBPs also have a positive role in Wnt signalling. However, it is also possible that the phenotype is indirect, that is, CtBP represses a negative regulator of the Wnt pathway. Similar to fly CtBP, if murine CtBP monomers also have a positive role in regulating Wg targets, then a gene knockin of monomeric mCtBP1 or mCtBP2 should rescue the defect in posterior structures of CtBP knockouts.

In fly embryogenesis, loss of CtBP results in dramatic disruption of segmentation, due to defects in the striped pattern of the primary pair rule genes (Poortinga *et al*, 1998; Nibu *et al*, 1998a; Strunk *et al*, 2001). Many of these defects can be explained by the requirement of CtBP to bind to gap gene transcription factors (e.g. Kr) and promote repression (Nibu *et al*, 1998a; Keller *et al*, 2000; Nibu and Levine, 2001; Strunk *et al*, 2001; Struffi *et al*, 2004). However, there are aspects of the *CtBP* mutant phenotype (e.g. loss of pair rule stripes (Poortinga *et al*, 1998; Nibu *et al*, 1998a) and genetic interactions (Poortinga *et al*, 1998; Phippen *et al*, 2000) that suggest that CtBP may have a positive role in regulating transcription. Testing whether $CtBP^{Mono}$ can rescue aspects of the CtBP segmentation phenotype may help determine whether CtBP has a direct role in activating transcription in regulatory hierarchies beyond the Wg pathway.

Materials and methods

Drosophila cell culture

Kc167 cell culture and RNAi-mediated knockdown were performed as reported previously (Fang *et al*, 2006). Cells (10^6/ml) were soaked in 10 μg dsRNA for 4 days, before seeding for transfections. Primers for dsRNA synthesis have been described elsewhere (Fang *et al*, 2006). Transient transfections were performed using Fugene 6 (Roche Applied Science) as per the manufacturer's instructions.

Plasmids and reporter assays

$pAcCtBP^{short}$ with 2x Flag tags at the C-terminus (kindly provided by Dr D Arnosti) was used for all rescue assays. Site-directed mutagenesis of $pAcCtBP^{short}$ (hereafter referred to as $CtBP^{WT}$) was used to introduce mutations in the dimerization interface to generate $CtBP^{Mono}$ (C134Y, N138R, R141A, R142A), $CtBP^{Basic}$ (E126R, E127R) and $CtBP^{Acidic}$ (R171E, R173E). The C-terminal HA-tagged versions were generated by replacing the 2x Flag tags of $pAcCtBP^{short}$ by 4x HA tags. The C-terminal V5-tagged versions were created by cloning the $CtBP^{WT}$ and mutant cDNAs into the *Kpn*I and *Not*I sites of *pAC 5.1 V5-His* (Invitrogen). *pGL3nkd-UpE1, pAcArm*, pAcGal4DBD, pAcGal4Arm*, pUAS-luc* and *pActinlacZ* constructs have been described elsewhere (Fang *et al*, 2006; Chang *et al*, 2008). *pACGal4Pygo* was constructing by cloning the dPygo ORF (815 amino acids) into the *Kpn*I and *Xho*I sites of *pACGal4DBD*. *pGL3CG6234* minimal WRE (*CG6234* WRE), a 539-bp fragment, was generated using PCR-based subcloning of a 617-bp region (−3220 to −2603 relative to the *CG6234* transcription start site) from the previously described *pCG6234* (Fang *et al*, 2006). Deletion of the region (−2603 to −1465) and an internal 80 bp deletion (−2860 to −2781) led to ∼4.5-fold increase in the activation of the WRE by Arm* in cell culture assays, and hence this reporter was used thereafter. For transgenic lines, cDNAs for the $CtBP^{WT}$ and mutants

with two C-terminal flag tags were subcloned into *pUAST* vector using the *Kpn*I and *Xba*I sites.

CG6234 WRE and *nkd-UpE1* reporter assays were performed by transiently transfecting 10–50 ng of the reporter and 150–500 ng CtBP expression plasmids in 2.5×10^5 cells/well. The *CG6234* WRE was activated using 100 μl of Wg-CM (derived from stable pTubWg S2 cells kindly provided by Dr R Nusse) or 50–100 ng of Arm*. For assays with *pUAS-luc*, 10 ng of the reporter and 5 ng of Gal4Arm* was used with 500 ng to 1 μg of the CtBP constructs. $CtBP^{Mono}$ protein was less stable compared with $CtBP^{WT}$ and to achieve equal expression levels, two times more of the $CtBP^{Mono}$ plasmid was transfected compared with $CtBP^{WT}$ in all assays. For all reporter assays, 5 ng of *pAcLacZ* was transfected for normalization and pAC5.1 (Invitrogen) or Gal4DBD to control for DNA amounts. Luciferase and LacZ assays were performed as described (Fang *et al*, 2006).

Drosophila genetics

Transgenic *UAS-CtBP* lines were generated using the injection facility at BestGene Inc. (Chino Hills, CA). w^{1118}, *GMR-Gal4*, *Ptc-Gal4*, *Dpp-Gal4*, *En-Gal4* and *Dll-lacZ* were obtained from Bloomington Stock Center. CtBP transgenes were analysed for their effect on the small eye phenotype of P[*GMR-Gal4*] P[*GMR-Arm**] flies as described previously (Cadigan *et al*, 2002; Parker *et al*, 2002). Experiments with *Ptc-Gal4*, *GMR-Gal4* and *En-Gal4* were carried out at 25°C and *Dpp-Gal4* at 27 or 29°C.

Immunoblots, immunostains and image quantification

For western blot analysis, anti-Flag (1:2500, Sigma), anti-V5 (1:5000, Invitrogen), anti-HA (1:1000, Roche) and anti-Tubulin (1:4000, Sigma) were used followed by HRP-conjugated anti-mouse or anti-rat IgG (Jackson Immunochemicals). Signal was detected using ECL kit (Amersham). Immunostaining of wing imaginal discs was performed as described previously (Fang *et al*, 2006) using anti-LacZ (1:1000, Abcam), anti-Wg (1:100) and anti-CtBP (1:1000). Alexa 488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes and Jackson Immunochemicals. Samples were examined using a Leica triple channel confocal microscope DM6000B-CS and processed using Adobe Photoshop 8.0.

For image quantification, total pixel intensity was determined using the Volocity Software 5.0 (Perkin-Elmer) after background subtraction. A region at the D/V boundary with the *Dll-lacZ* expression was selected in the anterior and posterior of the wing imaginal disc (Supplementary Figure S1). The mean pixel intensity of the posterior was normalized to the anterior.

Co-IP and ChIP

For co-IPs, $6–10 \times 10^6$ Kc cells were seeded with 1 μg pAcCtBP/10^6 cells for 3 days before harvesting. Cells were resuspended in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.9, 1% CHAPS, 10% glycerol, 0.1 mM EDTA with complete mini-EDTA free protease inhibitor cocktail, (Roche) and sonicated thrice on ice in pulses of 6 s. An alternate lysis protocol was used for experiments in Figure 7. Cells were resuspended in lysis buffer (100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 0.1 mM EDTA with complete mini-EDTA free protease inhibitor cocktail, (Roche) and incubated at 4°C for 1 h. Lysates were pre-cleared using Protein A/G sepharose beads. Total protein concentration was measured using the *DC* protein assay (Bio-Rad). Lysates corresponding to 3 mg total protein was used for each IP. In all, 15% of this lysate was saved as input. The remainder was incubated with 5 μg primary antibody for 2 h at 4°C followed by incubation with Protein A/G sepharose beads for 30 min at 4°C. The antibody–antigen complexes were washed four times with lysis buffer and eluted in 60 μl of Laemmli sample buffer for western blot analysis. Results shown are representative of at least two independent experiments.

ChIP analysis was performed as described previously (Fang *et al*, 2006). Briefly 3×10^6 cells and 10 μl of anti-CtBP antisera were used for every pull down and precipitated DNA subject to quantitative RT–PCR. Data are expressed as a percent of the input DNA. Specific primer pairs for the *UPE* and *ORF* correspond to *N*#1 and *N*#0 in the *nkd* locus as reported elsewhere (Fang *et al*, 2006; Chang *et al*, 2008).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: CB and JLC carried out the Drosophila cell culture experiments; CB and KMC carried out the Drosophila developmental genetics; CB, DLA and KMC designed the experiments and CB and KMC wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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