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CBP/p300 are bimodal regulators of Wnt signaling

Jiong Li, Chris Sutter, David S Parker, Timothy Blauwkamp, Ming Fang[1] and Ken M Cadigan*

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

Many Wnts influence cell behavior by a conserved signaling cascade that promotes the stabilization and nuclear accumulation of β-catenin (β-cat), which then associates with TCF family members to activate target genes. The histone acetyltransferase CREB binding protein (CBP) can bind to TCF and inhibit Wnt signaling in *Drosophila*. In contrast, studies in vertebrates indicate a positive role for CBP and the closely related protein p300 as β-cat binding transcriptional co-activators. We address this discrepancy by demonstrating that in addition to its negative role, CBP has an essential positive role in Wnt signaling in flies. CBP binds directly to the C-terminus of Armadillo (Arm, the fly β-cat) and is recruited to a Wnt-regulated enhancer (WRE) in a Wnt- and Arm-dependent manner. In a human colorectal cancer cell line, we show that CBP and p300 can inhibit Wnt signaling and demonstrate that human p300 can bind directly to TCF4 *in vitro*. Our results argue that CBP/p300 has an evolutionarily conserved role as a buffer regulating TCF-β-cat/Arm binding. Subsequent to this interaction, it also has an essential role in mediating the transactivation activity of β-cat/Arm.

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Introduction

The Wnt/β-catenin (β-cat) pathway is a signaling cascade that is highly conserved from cnidarians to humans (Cadigan and Nusse, 1997; Guder *et al*, 2006). During development, this pathway is used to control a variety of cell fate decisions (Cadigan and Nusse, 1997; Logan and Nusse, 2004). Misregulation of Wnt/β-cat signaling plays a causal role in several types of human cancers (Polakis, 2000), as well as in defects in bone density and vascular defects of the eye (Logan and Nusse, 2004).

The level of Wnt/β-cat signaling revolves around the stability and cellular location of β-cat/Armadillo (Arm; the fly β-cat). In the absence of Wnt stimulation, there is a small pool of cytosolic β-cat/Arm due to constitutive phosphorylation by a complex containing Axin, the adenomatous polyposis coli (APC) protein and glycogen synthase kinase 3 (GSK3; Ding and Dale, 2002). Phospho-β-cat is then targeted to the ubiquitin/proteosome degration pathway (Daniels *et al*, 2001). Upon Wnt stimulation, the Axin/APC/GSK3 complex is antagonized, causing the accumulation of hypophosphorylated β-cat/Arm, which translocates into the nucleus where it complexes with transcription factors, most notably members of the TCF family of DNA-binding proteins (Roose and Clevers, 1999).

Without β-cat/Arm, TCFs are thought to function as repressors of Wnt target gene expression, in part by interacting with transcriptional corepressors of the Groucho/TLE (Gro) family (Cavallo *et al*, 1998; Roose *et al*, 1998). β-cat is thought to displace Gro from TCF through competitive binding (Daniels and Weis, 2005). In addition to relieving TCF repression, β-cat/Arm is thought to activate Wnt target gene expression by recruiting additional proteins to TCF-bound chromatin. The N-terminal portion of β-cat/Arm binds Legless (Lgs, called BCL9 in vertebrates) and Lgs/BCL-9 acts as an adaptor between β-cat/Arm and Pygopus (Pygo), which promotes transcriptional activation (Kramps *et al*, 2002; Thompson, 2004; Hoffmans *et al*, 2005). The C-terminus of β-cat/Arm has been shown to bind to several transcriptional coactivators, including Hyrax/Parafibromin (Mosimann *et al*, 2006) and the chromatin remodeler Brg-1 (Barker *et al*, 2001). These interactions contribute to the ability of TCF/β-cat/Arm to activate Wnt target genes, supporting the model that β-cat/Arm converts TCFs from repressors into transcriptional activators (van Es *et al*, 2003; Parker *et al*, 2007).

Despite the strong conservation of the Wnt/β-cat signaling pathway between invertebrates and vertebrates, some important differences have been noted. For example, the histone acetyltransferase (HAT) Creb-binding protein (CBP) has been shown to be a negative regulator of Wnt signaling in flies (Waltzer and Bienz, 1998), but positively regulates the pathway in vertebrates (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000).

Mutations in the *nejire* (*nej*) gene, which encodes *Drosophila* CBP (Akimaru *et al*, 1997), had elevated levels of Wingless (Wg, a fly Wnt) signaling in the embryo and suppressed loss of Wg signaling phenotypes in the developing wing (Waltzer and Bienz, 1998). CBP was found to bind directly to the HMG domain of TCF and acetylate it on a conserved lysine in its N-terminal domain, reducing TCF's ability to bind to Arm. The data support a model where CBP negatively regulates TCF-Arm interaction, and thus Wg signaling, by binding and modifying TCF (Waltzer and Bienz, 1998).

In contrast to flies, the data from vertebrate systems support a positive role for CBP and the closely related HAT, p300, in Wnt signaling. Expression of either gene augments β-cat activation of reporter genes and inhibition of these genes reduces TCF reporter gene activity (Hecht *et al*, 2000;

*Corresponding author. Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University Avenue, Ann Arbor, MI 48109-1048, USA. Tel.: +1 734 936-3246; Fax: +1 734 647 0884; E-mail: cadigan@umich.edu
[1]Present address: Department of Genetics and Developmental Biology, Southeast University Medical School, Nanjing 210009, China

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Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000). These HATs can bind β-cat *in vitro* (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000), and are recruited to Wnt-responsive elements (WREs) in the *Cyclin D2*, *c-Myc* and *survivin* genes upon activation of Wnt/β-cat signaling (Kioussi *et al*, 2002; Ma *et al*, 2005; Sierra *et al*, 2006). Inhibition of CBP using siRNA or a chemical inhibitor that disrupts β-cat-CBP binding (Emami *et al*, 2004) was found to block Wnt activation of *survivin* gene expression (Ma *et al*, 2005). These data are consistent with the view that β-cat recruitment of CBP/p300 to WREs is required for transcriptional activation. In addition, p300 has also been shown to bind to a specific isoform of TCF4 and act synergistically to activate TCF reporters (Hecht and Stemmler, 2003).

In this study, we explore this controversy by re-examining the role of CBP in Wnt signaling in *Drosophila*. As previously reported (Waltzer and Bienz, 1998), we find evidence for CBP playing an inhibitory role in Wg signaling. However, we also demonstrate that CBP is required for activation of Wg targets, both in cell culture and wing imaginal discs. Fly CBP can bind directly to and interact functionally with the C-terminal half of Arm, and CBP is recruited to a WRE in a Wg- and Arm-dependent manner. In addition, we demonstrate that human p300 can bind to TCF4, and find that siRNA reduction of p300 and CBP leads to an elevation of Wnt signaling in a human colorectal cancer cell line. Our data support the view that the relationship between CBP/p300 and the Wnt pathway is evolutionarily conserved between flies and vertebrates. These HATs act as a buffer to regulate TCF-β-cat/Arm interaction, but have an additional role as β-cat/Arm binding transcriptional coactivators.

Results

Overexpression of CBP can repress or activate Wg signaling depending on the context

We identified *CBP* in a misexpression screen where the Rorth collection of EP insertions (Rorth *et al*, 1998) was crossed to P[*GMR-Gal4*]/P[*UAS-wg*] (GMR/wg) flies, which have a severe reduction in adult eye size (Parker *et al*, 2002). Two EP transposons inserted just 5′ to the *nej* gene, which encodes the only fly CBP (Akimaru *et al*, 1997), were found to be slight but significant suppressors of the GMR/wg phenotype (data not shown). A P[*UAS-CBP*] line also suppressed GMR/wg, indicating that CBP was the gene responsible for inhibiting the Wg pathway.

To extend these findings, the effect of CBP expression was examined in the developing wing. Wg is expressed in a narrow stripe along the dorsal/ventral (D/V) boundary of the wing imaginal disc, where it activates proneural genes such as *senseless* (*sens*) (Parker *et al*, 2002) and specifies wing margin in the adult wing (Couso *et al*, 1994). Expression of CBP at the D/V boundary of the wing disc causes notches in the adult wings that are characteristic of a loss of Wg signaling (Figure 1B). Expression of CBP in a stripe perpendicular to the D/V boundary, via a Decapentaplegic (Dpp)-Gal4 driver causes a loss of Sens (Figure 1G), consistent with a block in Wg signaling. However, Wg expression was also consistently reduced (Figure 1F), as was Cut expression (data not shown), suggesting a block in Notch signaling (Micchelli *et al*, 1997). When the animals were reared at 18°C, when Gal4 is less active (Rorth *et al*, 1998), loss of Sens without loss of Wg expression was observed at a low frequency (12%; $n=74$), with the remainder having a wild-type pattern (55%) or a loss of both Sens and Wg (33%; data not shown). These results suggest that at low levels of expression, CBP can inhibit the Wg target Sens without effecting Wg expression, but CBP also has an inhibitory affect on Wg expression, probably due to reduced Notch signaling.

To examine further the relationship between CBP and Wg signaling, a mutant version of CBP containing point mutations in the HAT catalytic site (CBP^{HATmut}; Ludlam *et al*, 2002) was expressed in the wing via Dpp-Gal4. The majority of these discs (56%; $n=39$) displayed a phenotype consistent with a loss of Wg signaling; a strong loss of Sens expression (Figure 1J) with no detectable reduction in Wg expression (Figure 1I). The remainder of the discs appeared normal (18%) or had loss of both Sens and Wg (26%). The Notch target Cut was largely unaffected in this background (data not shown). Expression of CBP and CBP^{HATmut} had different effects on another readout of Wg signaling, Distal-less (Dll), which is activated by Wg in broad domain centered on the D/V stripe (Zecca *et al*, 1996; Neumann and Cohen, 1997). Wild-type CBP did not alter the Dll pattern, even in discs where Wg expression was inhibited (Figure 2A–C). In contrast, CBP^{HATmut} caused a consistent (75%; $n=20$) reduction in Dll expression but had no effect or slightly expanded Wg expression (Figure 2D–F). These results suggest that CBP and CBP^{HATmut} are interacting with the Wg pathway through different mechanisms, with the Hat mutant possibly acting like a dominant-negative, consistent with a positive role for CBP in Wg signaling.

To explore the relationship between CBP and Wnt signaling in cell culture, we coexpressed a constitutively active, hypophosphorylated form of Arm (Arm*; Freeman and Bienz, 2001) with fly CBP in human embryonic kidney 293 (293) or *Drosophila* Kc167 (Kc) cells. The Topflash reporter, containing three TCF-binding sites upstream of the *c-fos* promoter (Korinek *et al*, 1997) was used in 293 cells and is activated by Arm* (Figure 3A). This construct is only expressed at low levels in *Drosophila* cells (M Fang and K Cadigan, unpublished observations), so the *c-fos* proximal promoter was replaced with that of the fly *Hsp70* gene. This reporter, called Dropflash, is activated by Arm* in Kc cells (Figure 3B). In the human cells, fly CBP increased Arm-dependent activation of the reporter by 2–3-fold (Figure 3A), similar to the effects reported with β-cat and p300/CBP (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000). However, no increase of Arm* activated Dropflash was observed in several experiments. Rather, there was a slight inhibition of reporter gene activation at higher CBP levels (Figure 3B). These data indicate that fly CBP can activate Arm transcriptional activity in some contexts but not others.

It is possible that in Kc cells, CBP's inhibitory activity masks the activation of Arm-mediated transcription. Since CBP inhibits the pathway by binding to TCF (Waltzer and Bienz, 1998), removing TCF from the system might uncover the positive effect of CBP on Arm. To do this, we utilized a construct expressing Arm fused to the DNA-binding domain of Gal4 (Gal4-DBD). Gal4-Arm can activate a UAS-luciferase (UAS-luc) reporter (Stadeli and Basler, 2005; Fang *et al*, 2006; Figure 3C). In contrast to Arm activation of Dropflash, coexpression of CBP consistently increased the transcrip-

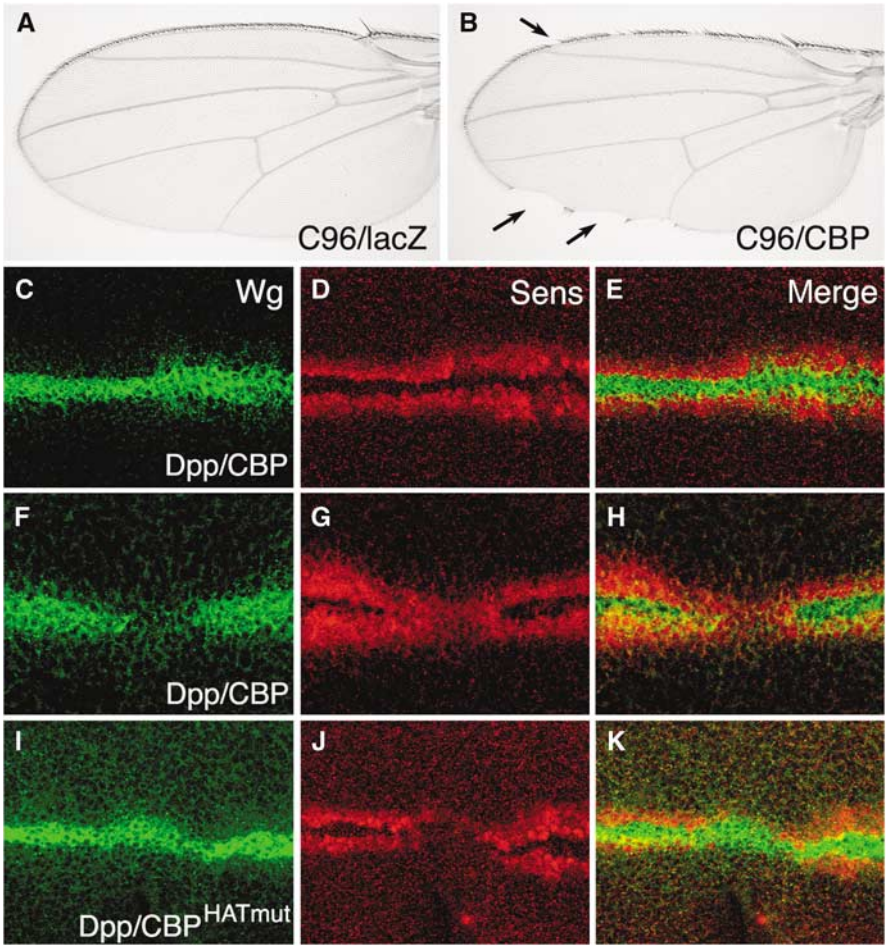

Figure 1 Misexpression of CBP and a CBP^{HATmut} inhibit Wg target gene expression. (**A**, **B**) Micrographs of adult wings containing the P[*C96-Gal4*] driver and either P[*UAS-lacZ*] (A) or P[*UAS-CBP*] (B). Misexpression of CBP leads to loss of wing margin (arrows in (B)). Expression of CBP^{Hatmut} produced a similar phenotype (data not shown). (**C–K**) Confocal images of late third-instar wing imaginal discs stained for Wg (green) or the Wg target Sens (red). (C–E) P[*Dpp-Gal4*]/P[*UAS-CBP*] disc reared at 18°C. Mild defects in Wg and Sens expression are sometimes observed. (F–H) P[*Dpp-Gal4*]/P[*UAS-CBP*] disc reared at 25°C. Most of the discs have a loss of Wg and reduction of Sens in the Dpp expression domain. (I–K) P[*Dpp-Gal4*]/P[*UAS-CBP^{Hatmut}*] disc reared at 29°C. The majority of the discs have no effect on Wg expression and a strong reduction in Sens expression.

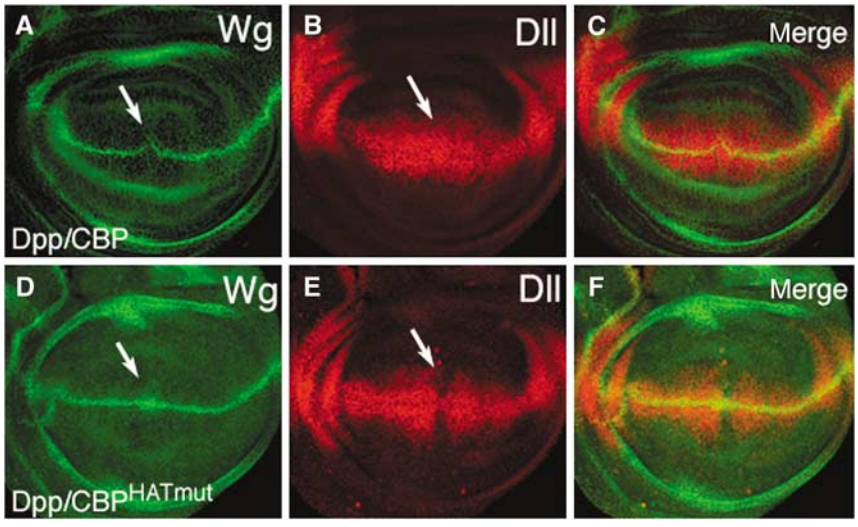

Figure 2 Misexpression of CBP and CBP^{Hatmut} have different effects on the Wg target Dll. Confocal images of late third-instar imaginal discs containing P[*Dpp-Gal4*] and either P[*UAS-CBP*] reared at 18°C (**A–C**) or P[*UAS-CBP^{Hatmut}*] reared at 29°C (**D–F**) stained for Wg (green) or Dll (red). Arrows indicate the location where the Dpp expression domain intersects the Wg D/V stripe. Although CBP expression causes a reduction of Wg expression in approximately half of the discs, the Dll pattern remains unchanged. In contrast, the large majority of discs expressing CBP^{HATmut} had normal or slightly expanded Wg expression and reduction of Dll expression in the Dpp expression domain.

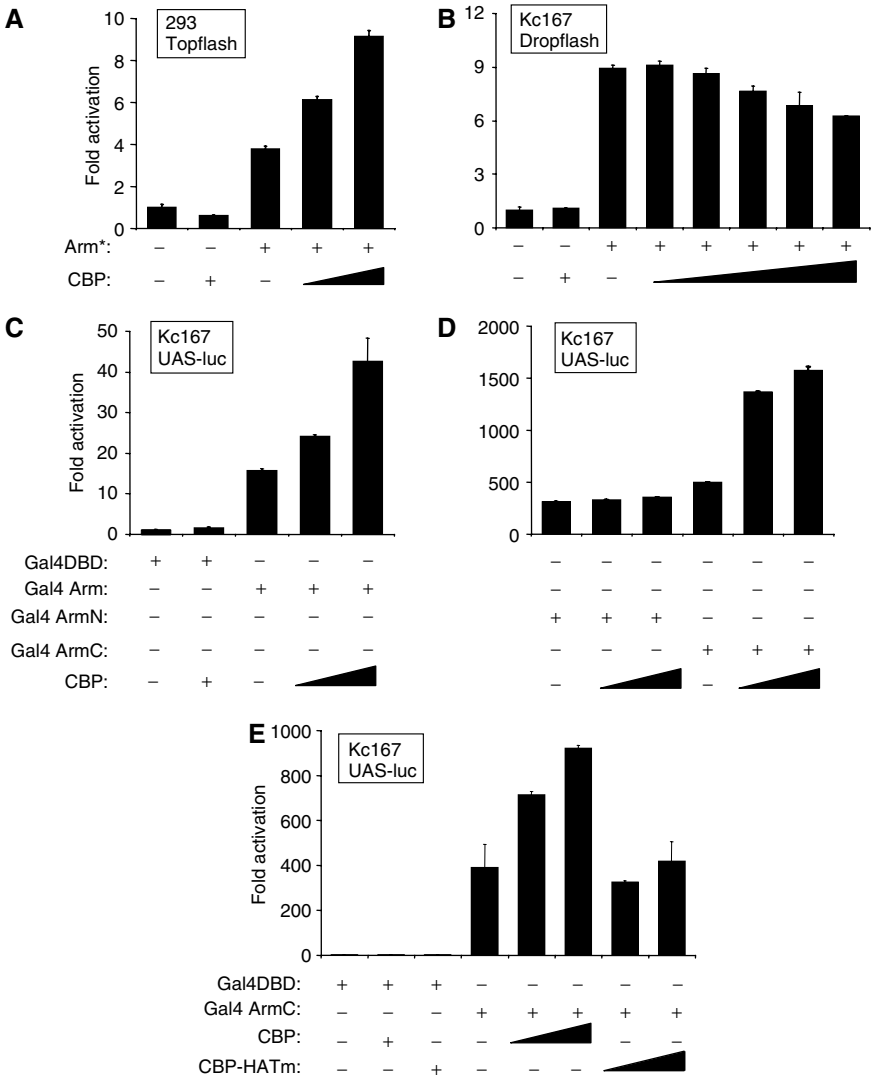

Figure 3 CBP augments Arm transcriptional activity in a context-dependent manner. (**A**) 293 HEK cells were transfected with plasmids expressing an activated form of Arm (Arm*; 20 ng) and fly CBP (200 or 400 ng) along with the Topflash luciferase reporter. CBP increases Arm activation of the Wnt reporter. (**B**) Fly Kc cells were transfected with Arm* (20 ng) and CBP (50, 100, 200 or 400 ng) expression constructs along with the Dropflash luciferase reporter gene. CBP had a slight inhibitory effect on the ability of Arm* to activate the Wg reporter. (**C–E**) Kc cells transfected with plasmids (20 ng) expressing the Gal4-DBD domain alone or Gal4-DBD fused to full-length Arm (Gal4-Arm), the N-terminal half of Arm (Gal4-ArmN; residues 1–428) or the C-terminal half of Arm (Gal4-ArmC; residues 429–815). CBP or CBP^{Hatmut} constructs were transfected as indicated at 20 or 50 ng. The Gal4 UAS-luciferase (UAS-luc) reporter gene was activated by Gal4-Arm, Gal4-ArmN and Gal4-ArmC, and CBP coexpression augmented this activation for Gal4-Arm and Gal4-ArmC but not Gal4-ArmN. CBP^{HATmut} did not effect Gal4-ArmC activation of UAS-luc. All transfections contained a lacZ expression plasmid, and luciferase activities were determined 48 h post-transfection and normalized against β-galactosidase activity. Values are the mean of duplicate experiments (standard deviations are indicated) and expressed as relative activity compared with cells transfected with the reporter alone.

tional activity of Gal4-Arm (Figure 3C). Thus, the positive effect of CBP expression on Arm is only apparent in Kc cells when the requirement for TCF is bypassed.

CBP interacts functionally and physically with the C-terminus of Arm

There are at least two regions of Arm/β-cat that mediate transcriptional activation. The C-terminus is sufficient for transcriptional activation but the N-terminal half also has this ability (van de Wetering *et al*, 1997; Hsu *et al*, 1998; Cox *et al*, 1999; Natarajan *et al*, 2001; Stadeli and Basler, 2005; Fang *et al*, 2006). Consistent with this, both halves of Arm fused to Gal4 DBD (Gal4ArmN and Gal4ArmC) are potent activators of UAS-luc (Figure 3D). However, coexpression of CBP had no effect on Gal4ArmN but did augment the ability of Gal4ArmC to activate the reporter (Figure 3D). Interestingly, CBP^{HATmut} had no effect on Gal4ArmC activity (Figure 3E), except an inhibitory one when CBP^{HATmut} is expressed at high concentrations (data not shown). The results demonstrate a functional interaction between CBP and the C-terminal half of Arm that is dependent on CBP's HAT activity.

To determine whether the functional interaction between Arm and CBP reflects a physical association, we examined the ability of the two proteins to associate in several assays. A bacterially produced glutathione-*S*-transferase (GST)-Arm fusion protein was able to pull down CBP from an extract of human cells expressing fly CBP (Figure 4A). No pull down

was observed from control extracts, or when a GST protein was used. Arm can be co-immunoprecipitated by CBP when both are coexpressed in Kc cells (Figure 4B). A co-immunoprecipitation of endogenous CBP and Arm was also observed when Kc cells are stimulated by conditioned media containing Wg protein (WCM; Figure 4C). These data demonstrate that CBP and Arm physically associate, although the interaction could be indirect.

As described above, CBP augments the activity of Gal4-ArmC but not Gal4-ArmN (Figure 3D). Consistent with this, GST-ArmC could pull down CBP from cell extracts, while GST-ArmN could not (data not shown). To determine whether the interaction between the C-terminal half of Arm and CBP is direct, fragments of CBP were fused to GST and incubated with ArmC produced by *in vitro* translation. Several CBP fragments at both the N- and C-termini could specifically interact with ArmC (Figure 4D). Thus, at least two domains of CBP can bind directly with the C-terminal portion of Arm, consistent with CBP directly acting with Arm to activate transcription.

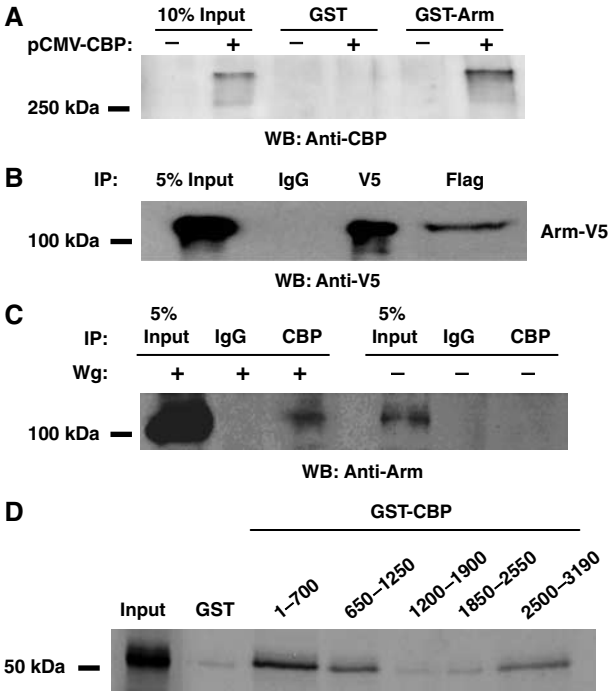

Figure 4 Arm interacts with CBP *in vitro* and *in vivo*. (**A**) Western blot with anti-CBP antisera demonstrating that GST-Arm, but not GST alone, is able to pull down fly CBP from extracts of 293 cells expressing CBP. (**B**) Arm and fly CBP interact when overexpressed in Kc cells. Cells were transfected with plasmids expressing V5-tagged Arm* and Flag-tagged CBP. Forty-eight hours post-transfection, cell extracts were prepared and immunoprecipitated with V5, Flag and control IgG antibodies and the precipitates were analyzed by Western blot with anti-V5 antibody. Flag precipitation pulled down a significant portion of Arm*-V5, compared with control IgG. (**C**) Endogenous Arm and CBP interact. Kc cells were treated with WCM or control media for 4 h before extract preparation. Proteins were immunoprecipitated with anti-CBP antisera or control IgG and Western blot were analyzed with anti-Arm antibody. CBP interacts with endogenous Arm in Wg-stimulated cells. (**D**) Arm and CBP interact directly *in vitro*. Bacterially expressed GST or GST–CBP fragments of the indicated residues were incubated with ^{35}S-Met-labeled Arm C-terminal fragment (residues 429–815). Precipitated proteins were analyzed together with 5% of the input material by SDS–PAGE and autoradiography. Three CBP fragments bound to the C-terminal fragment of Arm, while fragments comprising residues 1200–2550 show similar binding as the GST-negative control. All experiments shown were performed multiple times, with similar results obtained.

CBP is required for activation of several endogenous Wg targets

To determine whether CBP is required for Wg activation of endogenous transcriptional targets, several genes activated by Wg signaling in Kc cells were examined. *Naked cuticle* (*nkd*) and *notum/wingful* (*notum*) are Wg antagonists whose expression is activated by Wg signaling in flies (Zeng *et al*, 2000; Gerlitz and Basler, 2002; Giraldez *et al*, 2002). Wg stimulation of Kc cells significantly induced the transcript levels of these genes (Figure 5A and B) (Fang *et al*, 2006). When CBP was depleted by RNA interference (RNAi), activation of both Wg targets was markedly reduced (Figure 5A and B). Similar results were obtained with *CG6234* (data not shown, which is directly activated by Wg signaling in Kc cells; Fang *et al*, 2006). A more dramatic block in Wg activation of these genes could be observed by increasing the dose or time of the CBP RNAi treatment, but this alters the growth of the Kc cells and the expression of housekeeping genes is also reduced. Under the CBP depletion conditions used in Figure 5, the levels of β-*tubulin*, *arm* and *TCF*

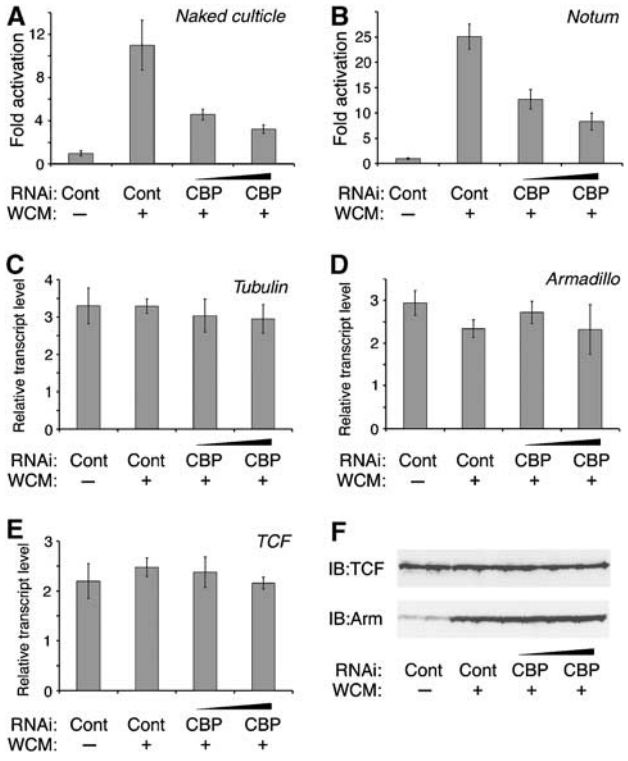

Figure 5 CBP is required for the transcriptional activation of Wg endogenous targets genes in Kc cells. (**A–E**) Cells were treated for 4 days with control dsRNA (10 μg/well) or different doses (7.5 and 10 μg/well) of dsRNA corresponding to *CBP*. Cells were then incubated for 5 h with control or WCM before transcript levels of *nkd* (A), *notum* (B), α-*tubulin* (C), *arm* (D) and *TCF* (E) were measured by quantitative RT–PCR as described in Materials and methods. Results for *nkd* and *notum* were normalized to the average of α-*tubulin*, *arm* and *TCF* expression, while the later three were normalized to total RNA. Wg activation of *nkd* and *notum* were reduced in *CBP*-depleted cells. (**F**) Western blot showing that induction of Arm protein and TCF protein levels were not affected by *CBP* depletion.

transcripts were unaffected (Figure 5C–E) and no decrease in TCF protein levels or the ability of Wg to stabilize Arm protein was observed (Figure 5F). Therefore, the defect in Wg activation of *nkd* and *notum* expression upon CBP knockdown is likely to be a conservative estimate of its requirement in the pathway.

Fly embryos that are zygotically mutant for a strong allele of *nej/CBP* have no detectable loss in Wg signaling, rather there is a significant increase in the pathway (Waltzer and Bienz, 1998). However, these embryos have a significant amount of maternally provided *CBP* (Ludlam *et al*, 2002). This maternal contribution cannot be removed, because it is required for oogenesis (Waltzer and Bienz, 1998). Thus, it is possible that a positive role for CBP in Wg signaling in the embryo has remained undetected because it is technically impossible to remove most of *CBP* gene activity in embryos.

To examine the requirement of *CBP* in the wing imaginal discs, somatic clones of the strong (nej^3) allele were induced in a *Minute/+* background (see Materials and methods for details). Under these conditions, small *CBP* mutant clones were obtained at a low frequency but displayed phenotypes consistent with a loss in Wg signaling. Wg expression at the D/V stripe was unaltered in *CBP* mutant clones (e.g. Figure 6F), but in half the clones ($n=8$), ectopic Wg expression was observed near the D/V stripe (e.g. Figure 6B). Away from the D/V boundary, 68% of the clones ($n=31$) had a low level of Wg expression inside the clone (data not shown). Despite this variable increase in Wg expression, there was a consistent decrease in the expression of the two Wg targets examined. A total of 91% of the clones near the Wg stripe ($n=11$) had a strong reduction in Dll expression (e.g. Figure 6C). Further way from the D/V boundary, 76% of the clones ($n=34$) had reduced Dll, while 12% had no observable defect (data not shown). The remaining 12% consists of four clones removed far from the Wg stripe, where Dll is normally not expressed. These had a slight elevation of Dll expression (compared with surrounding tissue) and all four also displayed ectopic Wg expression. Seven clones were examined for Sens expression: one showed a partial loss of Sens, two clones a stronger loss (data not shown), while the remaining four had a complete loss of Sen expression (e.g. Figure 6G). Overall, these data support a strong requirement for CBP in Wg activation of Dll and Sen.

Because it was so difficult to obtain nej^3 clones and the ectopic expression of Wg within these clones raised the possibility that cells lacking CBP were undergoing programmed cell death (Huh *et al*, 2004; Perez-Garijo *et al*, 2004; Ryoo *et al*, 2004), an alternative method of reducing CBP activity was utilized. A UAS line expressing a CBP hairpin known to produce the RNAi effect (Kumar *et al*, 2004) was driven in the posterior half of the wing pouch using the Engrailed (En)-Gal4 driver. A total of 45% of the discs examined ($n=20$) had an intermediate reduction of Sens and Dll, while the remaining 55% exhibited a stronger loss of these Wg targets (e.g. Figure 6J and K). In all cases, Wg expression was unaffected or slightly expanded (e.g. Figure 6I). The RNAi experiments and clonal analysis strongly support a positive role for CBP in Wg signaling in the developing wing.

CBP is recruited to a WRE in a Wg and Arm-dependent manner

Since *CBP* is required for activation of Wg targets and can bind directly to Arm, it is possible that CBP is recruited to WREs by Arm. We examined a region of the *nkd* intron,

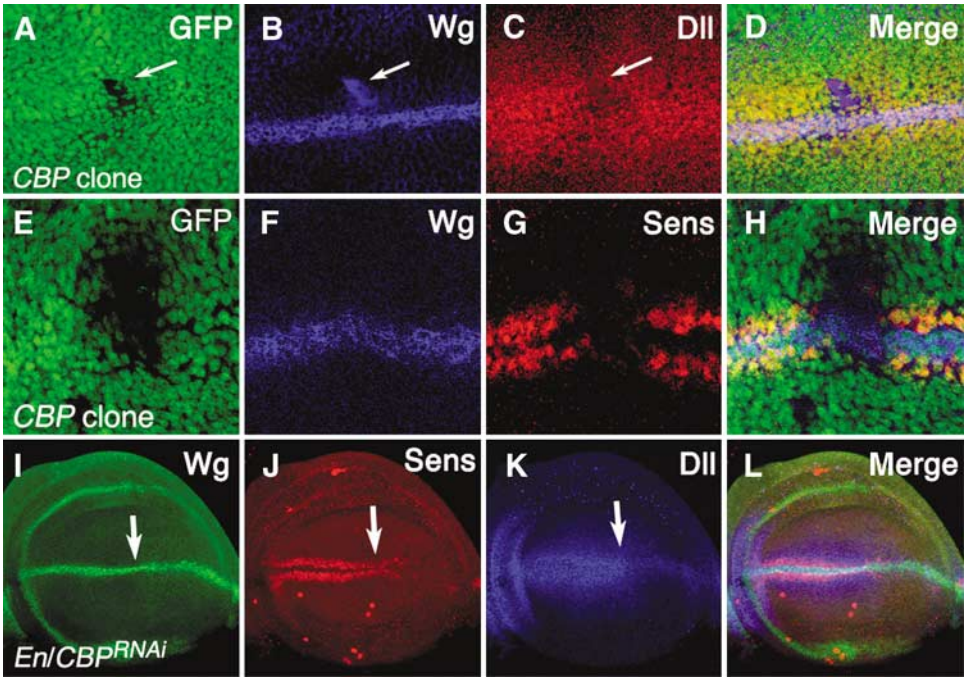

Figure 6 *CBP* is required in the wing imaginal discs for Wg signaling. Confocal images of late third-instar wing imaginal discs. (**A–H**) nej^3 mutant clones stained for the clonal marker GFP (A, E), Wg (B, F), Dll (C) and Sens (G). (A–D) In this *CBP* mutant clone, Wg expression is upregulated but expression of the Wg target Dll is greatly reduced (white arrows). (E–H) In this *CBP* mutant clone, Wg expression is normal but the Wg target Sens in not expressed. (**I–L**) P[*En-Gal4*]/P[$UAS\text{-}CBP^{RNAi}$] wing disc stained for Wg (I), Sens (J) and Dll (K). The En expression domain is to the right of the white arrows. Wg expression is unaffected by *CBP* depletion but Sens and Dll expression are greatly reduced.

approximately 5 kb downstream of the transcription start site, which we recently reported was bound by TCF, using chromatin immunoprecipitation (ChIP) (Fang *et al,* 2006). TCF binds to this region to a greater degree compared with other parts of the *nkd* locus, such as the *nkd* ORF (Figure 7A). In the absence of Wg stimulation, Arm is bound to this region at background levels (Figure 7B). However, addition of WCM for 4 h caused a marked increase in Arm binding (Figure 7B). As reported previously (Fang *et al,* 2006), a significant increase in TCF binding was also observed (Figure 7A). A 420 bp fragment encompassing this region and containing five putative TCF binding sites was fused upstream of the *hsp70* core promoter driving *luciferase* (*Nkd-luc*). This construct was activated 30-fold by cotransfection with Arm* (Figure 7C). When all five TCF sites were mutated, this activation was abolished (Figure 7C). These data strongly support that the stretch of DNA identified by ChIP and reporter gene analysis is a *bona fide* WRE directly regulated by the Wg/Arm pathway.

To determine whether CBP was also recruited to the *nkd* intronic WRE, ChIP was performed using CBP antisera. This antisera specifically recognized CBP, as judged by Western blot of Kc cells with or without CBP RNAi and immunostaining on wild-type cells or *nej*^{3} embryos (data not shown).

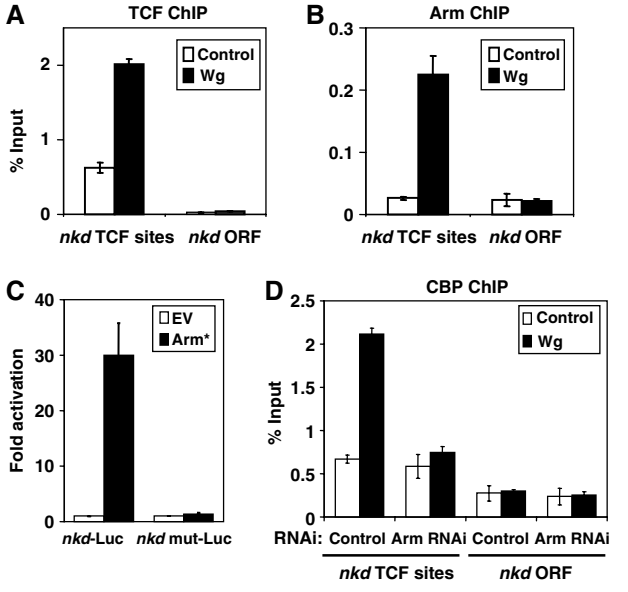

Figure 7 CBP is recruited to the WRE of the *nkd* gene in a Wg- and Arm-dependent manner. (**A, B**) ChIP using antibodies against TCF (A) and Arm (B) demonstrate enhanced binding to a cluster of TCF binding sites in the *nkd* intron (compared with the *nkd* ORF) in Kc cells stimulated with WCM for 4 h. (**C**) Kc cells were transfected with an Arm* expression plasmid and an *hsp70* luciferase reporter containing a 420 bp fragment of the *nkd* intron (*nkd*-luc) or the same sequence with five predicted five TCF destroyed by site-directed mutagenesis (*nkd*mut-luc). Reporter gene activity was assayed as described in Figure 3 and Materials and methods. Arm* activated *nkd*-luc 30-fold but did not activate *nkd*mut-luc. (**D**) CBP is recruited to the *nkd* WRE in a Wg and *arm*-dependent manner. Cells were transfected with control or *arm* dsRNA and cultured for 4 days before treatment with control or Wg-CM for 4 h before lysis and ChIP analysis with anti-CBP antisera. Precipitated DNA were purified and detected by Q-PCR using primers specifically against the *nkd* WRE or ORF as described in Materials and methods. Values are the mean of duplicate precipitations (±standard deviations) and the data is expressed as percentage of input DNA.

Binding of CBP was consistently enhanced 3–4-fold by Wg stimulation (Figure 7D). In the absence of Wg stimulation, the ChIP signal at the WRE was still reproducibly higher than at the ORF, suggesting it was present on the WRE. This signal was not reduced in cells depleted of Arm via RNAi. However, the Wg-dependent increase in CBP binding to the WRE was abolished by Arm RNAi (Figure 7D). CBP appears to occupy the *nkd* WRE in the absence of Wg signaling, but increases its occupancy upon Wg signaling in an Arm-dependent manner.

CBP and p300 repress Wnt signaling in a human colorectal cancer cell line

The data on *Drosophila* cells and imaginal discs clearly indicate a positive role for CBP in Wnt signaling, in addition to the inhibitory role previously described (Waltzer and Bienz, 1998). This raised the possibility that CBP/p300 also plays a negative role in vertebrate Wnt signaling. To test this, we examined whether the HMG domain of TCF4 could directly bind to a p300 fragment that contains the second cysteine/histidine-rich (CH2) domain. These portions of fly TCF and CBP had previously been shown to bind each other *in vitro* (Waltzer and Bienz, 1998). The TCF4 HMG domain can specifically precipitate the p300 fragment, compared to GST alone (Figure 8A).

The interaction between p300 and TCF4 suggested that p300/CBP could functionally repress Wnt signaling in a

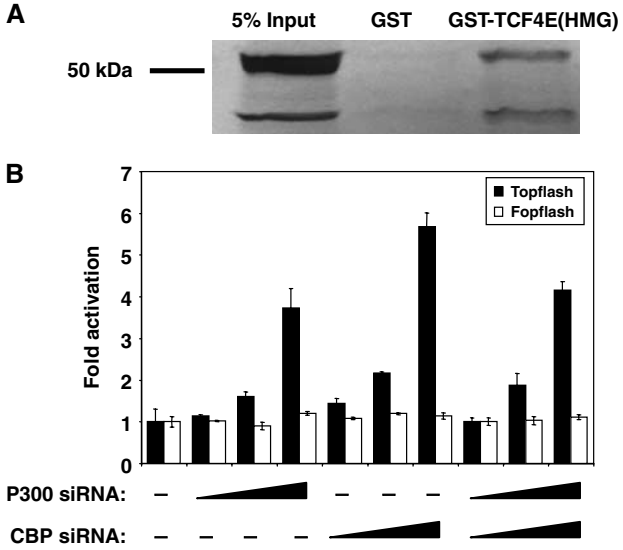

Figure 8 p300 binds to the HMG domain of TCF4E and CBP/p300 represses Wnt signaling in human cells. (**A**) The TCF4E HMG domain directly binds to p300. Bacterially expressed GST or GST-TCF4 (HMG domain; residues 326–396) fusion protein were incubated with a radiolabeled fragment of human p300 (residues 1254–1899). After pull down and washing, proteins retained by GST or GST-Arm were analyzed together with 5% of the input material by SDS–PAGE and autoradiography. (**B**) Human SW480 cells were transfected with the either the Topflash or Fopflash reporters, along with increasing amounts (100–500 ng) of pSuper-CBP^{RNAi} or pSuper-p300^{RNAi} constructs (empty pSUPER vector was used to normalize the amount of DNA transfected), which express short hairpins corresponding to each gene. CBP or p300 depletion increases TOPFLASH but not FOPFLASH reporter activity. Transfected cells were assayed for luciferase as described in Figure 3 legend and Materials and methods.

manner similar to its fly counterparts. To test this, used SW480 cells, which contain mutations in the APC gene causing constitutive Wnt signaling (Polakis, 2000). The cells were transfected with increasing amounts of plasmids expressing short RNA hairpins corresponding to CBP and p300 and the Topflash and Fopflash reporters (these hairpins have distinct sequences; see Materials and methods). siRNA knockdown of either CBP or p300 caused a significant increase in Topflash reporter activity but had no effect on the control reporter Fopflash (Figure 8B). The increase in reporter gene expression in CBP siRNA-treated cells could not be rescued with expression of p300 or fly CBP. Rather expression of these genes usually caused an additional increase in Topflash activity (data not shown). While the activation of Topflash by CBP and p300 siRNA could be due to off-target effects, we suggest that the rescue experiment may be complicated due to the fact that CBP both represses and activates TCF-β-cat transcriptional activity.

Discussion

CBP/p300 are bimodal regulators of Wnt signaling in both flies and vertebrates

The controversy over CBP/p300 action in Wnt/β-cat signaling is complicated by the fact that the conflicting models are both supported by strong evidence. In *Drosophila*, loss-of-function genetics clearly supports a negative role for fly CBP in the pathway, which was buttressed by the finding that CBP can bind and acetylate TCF, reducing its ability to bind Arm (Waltzer and Bienz, 1998). In vertebrate systems, expression of CBP or p300 can augment the ability of β-cat to activate reporter gene expression, and β-cat and CBP/p300 have been shown to interact directly (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000). Consistent with this, an increase in β-cat levels (by lithium treatment) results in CBP/p300 recruitment to WREs (Kioussi *et al*, 2002; Ma *et al*, 2005; Sierra *et al*, 2006) and inhibition of CBP-β-cat interaction reduces the ability of Wnt/β-cat signaling to activate transcriptional targets (Emami *et al*, 2004; Ma *et al*, 2005). The data support a view that CBP is a repressor of the pathway in flies and an activator in vertebrates.

This report resolves part of the discrepancy by providing strong support for a positive role for CBP in fly Wg signaling. Loss of CBP results in a dramatic reduction in the ability of Wg to activate transcriptional targets in fly cell culture (Figure 5) and the wing imaginal disc (Figure 6). This positive role for CBP was probably previously missed because technical reasons prevented the complete removal of *CBP* gene activity in the embryo. Our genetic results are complemented by our data showing that the C-terminus of Arm can bind to CBP both *in vitro* and in cells (Figure 4). Consistent with this, CBP is recruited to a WRE in a Wg- and Arm-dependent manner (Figure 7D). Thus, our data indicate a direct, essential requirement for CBP in Wg signaling in flies.

In addition to a positive role for CBP in Wg signaling, we also found evidence supporting the negative role previously described (Waltzer and Bienz, 1998). Expression of CBP inhibits Wg signaling in the *Drosophila* eye and wing (Figure 1 and data not shown), and a slight inhibition was also observed using a TCF reporter in fly cell culture (Figure 3B). In addition, CBP occupies a TCF-bound WRE in the *nkd* locus, even in the absence of Wg signaling (Figure 7D). These data are consistent with the model proposed by Waltzer and colleagues, stating that CBP negatively regulates Wg signaling through direct interaction with TCF (Waltzer and Bienz, 1998).

A negative role for p300 and CBP in mammalian Wnt signaling is suggested by our finding that human p300 can bind directly to human TCF4 (Figure 8A). This interaction was observed with the HMG domain of TCF4 and a fragment of p300 containing the CH2 domain, the same regions that interact with the fly counterparts (Waltzer and Bienz, 1998). In addition, siRNA knockdown of CBP and p300 causes a significant increase in activation of a Wnt reporter genes in human SW480 cells (Figure 8B). These results suggest that human p300 and CBP repress TCF-β-cat gene activation through interaction with TCF4 in a colon cancer cell line.

Taken together, our results suggest a model where CBP/p300 both represses and activates TCF-β-cat/Arm transcriptional activation. We envision that this bimodal regulation of Wnt signaling by CBP/p300 acts with other factors to set sharp thresholds of gene activation by nuclear β-cat/Arm. Depending on the cell type and level of nuclear β-cat/Arm, reduction of CBP/p300 will lead to either an increase, for example, the fly embryo (Waltzer and Bienz, 1998) and SW480 cells (Figure 8B), or a decrease, for example, Kc cells (Figure 5) or wing imaginal discs (Figure 6), in TCF transcriptional activity. This model can also explain why expression of CBP/p300 can lead to either activation (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000) or repression (Figures 1A–H and 3B) of Wnt signaling.

Mechanism of CBP/p300 activation of Wnt signaling

Our working model is that Wnt signaling results in increased recruitment of CBP/p300 to WREs through direct interaction with β-cat/Arm. The N-terminal portion of CBP and p300 can bind β-cat (Labalette *et al*, 2004) and two domains, a transcriptional adaptor putative zinc finger (TAZ finger, sometimes called the CH1 domain) and a KIX domain, have been shown to be sufficient for the interaction (Sun *et al*, 2000; Takemaru and Moon, 2000). A TAZ finger in the C-terminal third of CBP/p300 (also called the CH3 domain) can also bind to β-cat (Daniels and Weis, 2002; Hecht *et al*, 2000; Miyagishi *et al*, 2000). In our studies with the fly proteins, fragments containing the N-terminal TAZ finger (residues 1–700) and the KIX domain (residues 650–1250) were positive for binding (Figure 4D). However, a fragment containing the C-terminal TAZ finger (residues 1850–2550) did not show binding in our assay. Rather, the extreme C-terminal bound Arm (Figure 4D). This region is not conserved with vertebrate CBP or p300. In agreement with the vertebrate studies (Hecht *et al*, 2000; Miyagishi *et al*, 2000; Sun *et al*, 2000; Takemaru and Moon, 2000; Daniels and Weis, 2002), we found that the C-terminal half of Arm was sufficient for binding to CBP (Figure 4D). Several groups also found binding between the N-terminal portion of β-cat and p300 (Sun *et al*, 2000) or CBP (Miyagishi *et al*, 2000). In our study, we did not observe either physical or functional interactions between the N-terminal half of Arm and CBP (Figure 3D and data not shown). Although all the data cannot be neatly reconciled, it appears that β-cat/Arm interacts with CBP/p300 at multiple sites on each binding partner.

What is the consequence of CBP/p300 recruitment to WREs? CBP and p300 possess intrinsic HAT activity and are thought to activate transcription by the acetylation of lysine residues in the N-terminal tails of H3 and H4 histone subunits (Grant and Berger, 1999). An increase in H3 acetylation was also correlated with CBP/p300 recruitment to the *cyclin D2* WRE (Kioussi *et al*, 2002), but this correlation was not observed at the *survivin* WRE (Ma *et al*, 2005). In addition, CBP and p300 have also been shown to acetylate lysines on β-cat (Wolf *et al*, 2002; Labalette *et al*, 2004; Levy *et al*, 2004). Acetylation of β-cat increases its affinity for TCF, suggesting that this could account for its ability to augment TCF transcriptional activation (Levy *et al*, 2004). Casting doubt on the importance of these modifications is the finding that p300 lacking HAT activity can still augment β-cat activation of a TCF reporter gene (Hecht *et al*, 2000).

In this report, we observed that expression of CBP^{HATmut} blocks Wg signaling in the wing imaginal disc (Figures 1J and 2E). HAT activity is thought to be required for the ability of CBP to repress the Wg pathway (Waltzer and Bienz, 1998). This suggests that the inhibition observed with CBP^{HATmut} is due to a dominant-negative effect on CBP activation, that is, the mutant CBP outcompetes endogenous CBP for binding to Arm. In cell culture, expression of CBP^{HATmut} failed to augment the transcriptional activity of a Gal4 fused to the C-terminal half of Arm (Figure 3E) and inhibited Gal4-ArmC activity when expressed at high levels (data not shown). These results support the notion that HAT activity is required for CBP promotion of Wg signaling. Further studies will be required to determine whether modification of H3/H4, TCF or other protein substrates by CBP contributes to activation of Wnt transcriptional targets.

Materials and methods

Plasmids

The constructs CMV-p300, and the Topflash/Fopflash reporters were a kind gift from A Hecht. pActin5.1-CBP was from S Smolik. cyclin D-luciferase (Tetsu and McCormick, 1999) was obtained from E Fearon. The *Drosophila*-specific TCF reporter Dropflash was made by replacing the *c-fos* promoter of Topflash with the fly *hsp70* minimum promoter. Quick change site-directed mutagenesis (Stratagene) was used to engineer Arm* (T52A/S56A) and CBP^{HATmut} (Y2160A/F2161A). The Gal4-Arm and UAS-luc constructs are as previously described (Fang *et al*, 2006). Expression vectors of Flag-CBP, $CBP^{HAT\ mutant}$ and Arm* were constructed in pActin5.1 vector by standard PCR cloning or subcloning. An Arm*-V5 vector using the V5 epitope present in pActin5.1 was also constructed. The pSuper-CBP and pSuper-p300 constructs for siRNA were constructed as according to the manufacturer's (Brummelkamp TR) instructions, using hairpins from the coding region of each gene (p300 is 5′-GCTTGATGAATGCAGCCAA-3′; CBP is 5′-TGCTG CAGGCGGTGCTGGA-3′). All prokaryotic vectors for GST fusions proteins were constructed in the pET42a vector (Novagene). Bacterial expression for 6his-Flag-Arm 428-C and 6his-Arm FL were constructed in PET28a vector (Novagene). A fragment of p300 (residues 1254–1899) was cloned into pBluescript for *in vitro* translation.

Cell culture, transfection and reporter gene assays

293 or SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FBS at 37°C in a 5% CO_2/95% air atmosphere. For transient transfections, 1 million 293 or SW480 cells were seeded in to 12-well plates 12 h before transfection. For 293 cells, Lubrofactimine 2000 was used as the transfection reagent according to the protocol provided by the manufacturer (Invitrogen). SW480 were transfected using the FuGENE6 transfection reagent (Roche Molecular Biochemicals). Kc cells were cultured and transfected as previously described (Fang *et al*, 2006). Luciferase and β-galactosidase activities of total cell lysates were determined using Luc-ScreenTM and Galacto-StarTM kits (Tropix). The values reported are the means and standard deviations of the results from two independent experiments.

Immunoprecipitations, Western blotting and immunostains

For each immunoprecipitation reaction, 5 million Kc cells were lysed in 500 μl of lysis buffer (1% Chaps, 20 mM HEPES, pH 7.5 and 140 mM NaCl) with the protease inhibitor cocktail from Roche for 60 min on ice. After centrifugation at 10 000 r.p.m. at 4°C, the supernatant was transferred into a new tube and incubated with antibody at 4°C for 1 h, followed by incubation with a 20 μl bed volume of protein A or protein G–Sepharose (Amersham Pharmacia) for 1 h. A 5 μl volume of mouse monoclonal anti-Arm (Hybridoma Bank, University of Iowa), mouse anti-V5 (Invitrogen) and mouse anti-Flag (Sigma) was used for immunoprecipitations. For precipitating endogenous CBP, 5 μl of affinity-purified chicken anti-dCBP antibody followed by 5 μg of rabbit anti-chicken IgY secondary antibody were used. Immunoprecipitates were washed four times with lysis buffer at 4°C. Proteins bound to the beads were eluted with SDS–loading buffer at 98°C for 2 min, subject to SDS–PAGE and then transferred to membrane, followed by Western blotting analysis using ECL plus kit (Amersham Pharmacia). Immunostaining of wing imaginal discs with mouse anti-Wg, rabbit anti-Dll and guinea pig anti-Sen was performed as previously described (Fang *et al,* 2006).

RNAi knockdown and Wg conditioned media treatment

Double-stranded RNA (dsRNA) corresponding to *CBP* and arm was synthesized as described (Fang *et al*, 2006). Fragments of both genes were amplified with oligos containing the T7 promoter and the following gene-specific sequences (CBP:5′-GGGTACGCCTCCT TACATACCCGC and 5′-CCGCCACAGCTGTCATCCATAAACTCC and Arm: 5′-ATGAGTTACATGCCAGCCCAGAATCGAA and 5′-CGATGG TGTGATAAGTTGTGCAGTGTTCCTA).

One million Kc cells were seeded in 12-well plate in *Drosophila* SFM (Invitrogen) in the presence of 10 μg specific dsRNA or control dsRNA. After culture at room temperature for 2 h, 5% of FBS was added and the cells were cultured for 4 days. WCM was prepared using stable *pTubwg* S2 cells, kindly provided by Dr R Nusse from Stanford University, and was typically concentrated to approximately 50-fold using a Centricon tube (Millipore) and stored at −80°C. Kc cells were treated with Wg-CM (10 μl/1 $\times 10^6$ cells) for 4–8 h before harvesting.

RT–PCR and Q-PCR assays

Total RNA of Kc cells was purified using RNAwiz RNA isolation reagent (Ambion) and cDNA was synthesized with oligo-dT primers using SuperScript (Invitrogen). Quantitative PCR (Q-PCR) was performed as previously described (Fang *et al,* 2006). Primer pairs for *notum* are 5′-GCTGCT CTGCGTGATCGTCTTC-3′ and 5′-TCTG GTGTTGGTGAACTCTCCTCC-3′; primer pairs for *nkd* are 5′-TAAAA TTCTCGGCGGCTACAA-3′ and 5′-CGCACCTGGTGGTACATCAG-3′. β-Tubulin 56D levels are used as a loading control as previously described (Fang *et al,* 2006). The values reported are the means and standard deviations of the results from two independent experiments.

ChIP

Kc cells (five million) were treated for 20 min with 5 mM dimethyl 3,3′-dithiobispropionimidate–HCl (DTBP) (Pierce) in PBS at room temperature, rinsed with 100 mM Tris–HCl, 150 mM NaCl (pH 8.0) and crosslinked with 1% formaldehyde in PBS at 37°C from 20 min. Total cell lysates were sonicated to generated 200–1000 bp DNA fragments. Immunoprecipitation was performed with specific antibody or control IgG (Upstate) using ChIP assay kit (Upstate). Promoter regions were detected by Q-PCR with specific primers. Primers pairs for NKD TCF cluster are 5′-TCAATCAGACGTCAGAGG TACCG-3′ and 5′-CTGATGGAAGAACCGTGTTGG-3′; primer pairs for NKD ORF are 5′-CCAGCATCGCTATCGACCA-3′ and 5′-GCGTCCT TCTCCTTTTCGCT-3′.

Drosophila genetics

The Rorth collection of EP elements (Rorth *et al*, 1998) was screened and as described previously (Parker *et al*, 2002). The P[*GMR-Gal4*] P[*UAS-wg*] and P[*GMR-Gal4*] P[*GMR-arm**] are as

described previously (Parker *et al*, 2002). The C96 Gal4 drivers was provided by J Krupp and Dpp-Gal4 was obtained from the Bloomington Stock Center. The P[*UAS-CBP*] and P[*UAS-CBP^{Hatmut}*] (Ludlam *et al*, 2002) were generously provided by S Smolik, as were the *nej^{1}* and *nej^{3}* alleles. The P[*UAS-CBP^{RNAi}*] transgenic stock (Kumar *et al*, 2004) was generously provided by J Duffy. The *nej* alleles were recombined onto a chromosome containing P[*FRT*]^{18A} by recombination as described (Xu and Rubin, 1993). Clones of *nej^{3}* were generated by mitotic recombination using *hsFLP* and a *P[FRT]^{18B}P[Ubi-GFP] RpS5^{2}* chromosome carrying a *Minute* mutation via a 1 h 37°C heat shock at 24–48 h after egg laying. During these experiments, two types of GFP-negative clones were obtained: 52% ($n=176$) were small in size and showed a high penetrance of defects consistent with a block in Wg signaling; the remainder were extremely large in size (usually occupying half the disc) and were phenotypically wild type. We believe these large clones are not the result of mitotic recombination (and hence not mutant for CBP). Rather, they could have arisen from an intrachromosomal loss of both the P[*Ubi-GFP*] transgene and the *RpS5^{2m}* mutation. This could occur if there was an additional P[*FRT*] insertion on the chromosome. Because we consider it impossible that these large clones are homozygous for *nej^{3}*, they were not included in the summary of the phenotypes described in the Results.

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