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The oligomeric state of CtBP determines its role as a transcriptional coactivator and corepressor of Wingless Targets

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the long time taken to get back to you with a decision - this was due to some delay in initial handling of the manuscript (caused by a very high submission rate at the time), followed by a delay in receiving the final referee's report. However, I do now have the comments from all three reviewers, which are enclosed below.

As you will see, all three referees express significant interest in your work, and - to varying extents - are in favour of publication. However, while referee 2 raises only relatively minor criticisms, referees 1 and 3 both have more serious concerns that would need to be addressed experimentally. The comments of referee 1 are rather specific and self-explanatory; addressing the question as to whether the dimer is competent to direct gene activation, or whether only the monomer participates in this, would be particularly important. Also critical, and as highlighted by this referee, would be a more detailed analysis of the oligomerisation status of endogenous CtBP in cells. Referee 3 raises rather broader concerns - as to how monomer vs. dimer state is regulated, and how, mechanistically, the two forms differentially affect transcriptional activity. Both these points are discussed quite extensively, but not really addressed experimentally, although you do state that you see no change in oligomerisation status upon Wnt signalling. I do recognise that both questions are challenging to address and that a complete answer to either likely lies well beyond the scope of this study, but any additional analysis you can provide in either or both of these directions would be very valuable.

In the light of the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments raised by the referees. In terms of referee 3's concerns, I suggest that the best way forwards would be for us to discuss this in more detail, either

by email or over the phone, once you have had the chance to look through the referees' comments and discuss with your co-authors. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this study by Bhambhani et al., two different actions of the gene regulator CtBP are probed. Previously this group studied two target genes that are regulated by Wingless signaling (via TCF/armadillo complexes) and CtBP. One gene, naked (nkd), is actively repressed by TCF in the absence of Wg signaling. CtBP is involved in this repression as its simultaneous knockdown with TCF synergistically relieves repression of transcription. The CG6234 gene is different in that activation by Wg is reduced with CtBP knockdown. The opposite effects of CtBP on nkd (relief of repression) and CG6234 (reduction of activation) are interesting and the current study asks why/how CtBP might contribute different activities to two Wnt target genes. The question addressed in this study is significant because CtBP is a broadly acting regulator and is highly conserved in many model systems. CtBP has important effects on mammalian Wnt signaling and the principles addressed here are just as applicable in these other settings. Overall, this is an important study and it generates a new experimental approach that will be useful to many groups (CtBP monomers and forced dimers). However, new mechanistic conclusions are somewhat limited and are not fully supported by the experiments as presented and should be strengthened for greater impact.

1. Monomer/dimer distribution in cells
The authors show that dimerization is necessary for the repression activities of CtBP. They are careful not to make similar statements for the monomer form since they have shown only that it is sufficient for participation in activation; they have not shown monomers are required (i.e. that dimers don't activate). Dimer-competent wildtype CtBP can rescue activation just as well as CtBP monomer mutant (Fig. 4C, D, and E). This implies that either monomers are not required (i.e. dimers can activate in some settings and repress in other contexts) or that a significant portion of CtBP wildtype exists as a monomer. Can the authors show the distribution of CtBP monomer/dimers in cells? It appears to this reviewer that free monomer might not be abundant since co-expression of the acid/basic monomers appear to stabilize the levels of each form (Fig.6C, lanes 1,3 ad Fig. 4E inset).

2. Endogenous target genes
i. The authors use a modified fragment of the CG6234 gene for their reporter assays, including an 80bp internal deletion to increase responsiveness to Wg. Also, the region of the naked locus used for reporter activity is from an upstream region previously shown by this group not to be bound by

endogenous CtBP or endogenous TCF (Fang et al. 2006). In light of these modifications and differences with past data, it is important to corroborate the findings from the reporter assays with chromatin immunoprecipitation at the corresponding endogenous sites.
ii. Likewise, RT-PCR for endogenous naked and CG6234 mRNA levels would test whether the reporter genes are faithfully mimicking effects on endogenous gene transcription.

3. Monomer recruitment versus monomer inactivity
i. The authors show that monomer CtBP does not support gene repression, but whether this inability is due to the fact that monomer cannot be recruited to regulatory sites, or whether it is recruited but not active is not addressed. The results shown in Figure 5C imply that monomer cannot be recruited for repression (monomer overexpression does not relieve repression under control RNAi conditions, meaning that it does not competitively displace endogenous dimers). A ChIP assay with tagged wildtype, tagged monomer, and/or tagged acidic/basic dimers would go far to test this part of the mechanism. That is, if monomer cannot be recruited to repression elements, then tagged acidic CtBP will only bind to regulatory sites in the presence of its basic heterodimer counterpart. In contrast, tagged acidic monomer and other tagged monomers should be capable of binding to sites for activation (CG6234) on their own.
ii. The authors show that acidic and basic heterodimers recapitulate CtBP repressor action on the Naked reporter construct. However, they do not test whether these monomer mutants work individually as activators for the CG6234 reporter. If acidic and basic monomers can activate CG6234 transcription, then co-expression of the mutants to reduce the monomer pool and form a large heterodimer pool, would test whether dimer formation interferes with activation of a CG6234 - a complementary experiment to the one above.

4. Minor comments
i. The authors state that DNA sequence context must influence CtBP action. However, CtBP monomers work to activate the artificial UAS-Luc reporter with Gal4-Arm fusion protein just fine. It does not appear that DNA sequence context plays a role in the activation part of CtBP action.
ii. The very nice phenotypes shown in Figure 2 shows that CtBP wildtype and CtBP monomers have different actions. On the other hand the Dll-LacZ reporter data in Figure 3 shows that they both activate the enhancer. Can the authors state, or otherwise discuss whether the majority of CtBP actions is dimer-mediated repression and that only a few genes (to date: CG6234, perhaps Dll) respond to CtBP activation?

Referee #2 (Remarks to the Author):

The authors address an important issue, as Wnt regulated transcription plays key roles in both normal development and cancer. A number of studies have ascribed important roles to the transcription factor CtBP in the transcriptional output of the Wnt signal, but these studies have left a somewhat confusing picture, as they support both positive and negative roles for CtBP. Here the authors use a nice combination of in vitro and in vivo studies, and combine this with a clever mutagenesis strategy to sort out this confusion. They clearly demonstrate that the oligomeric state of CtBP determines whether it acts as a repressor or activator. The data are clear and convincing and the conclusions well supported. This work will be of broad interest to those studying Wnt signaling and transcriptional regulation in both flies and mammals. I only had a few relatively minor suggestions.

Introduction. P. 5. Is it known whether the repression role of CtBP requires enzymatic activity?

Fig. 3 would be clearer if both single channel images were shown in greyscale rather than color.

p. 9. The authors need to be a bit more detailed in their interpretation of the data in Figs 2 and 3. In the eye (Fig. 2), expression of CtBP wildtype and CtBP mono have opposite effects were as in the wing disc (Fig. 3) they have similar effects. While this is plausible, the authors should acknowledge this and provide a possible explanation.

p. 11. The authors should mention in the first paragraph that their earlier data suggest CtBP can bind Arm, as this is necessary to understand how it can effect Gal4-Arm*. It also would be worth mentioning this in the Introduction. Now, it only comes up late in the Discussion.

Fig. 4B. It would be nice to also include a "blowup" of the 0 ng Arm* data, to show whether the RNAi treatments have an effect on "basal levels"

Fig. 5. Why are the effects of CtBP WT and CtBP Mono opposite, instead of one simply being inactive in this assay?

p. 13. I thought it was a bit of an overstatement to call the V5 tagged protein "HIGHLY unstable". Also, was the data in Fig. 6D generated with tagged forms of the protein?

Referee #3 (Remarks to the Author):

This is a potentially interesting study that defines the molecular mechanism that enables CtBP to regulate the transcription of Wg targets in a gene-specific manner. The authors successfully demonstrate that dimeric CtBP represses the transcription of Wg targets while monomeric CtBP acts as a transcriptional activator.

The experiments described are straightforward and well-designed. The data are sound and appear to support the authors' conclusions. The results highlight the importance of the oligomeric state of CtBP in the Wg target-specific transcriptional regulation, and may be of interest to the readership of the EMBO Journal. However, my major concerns are: 1) upstream signaling that switches between monomeric and homodimeric CtBP, and 2) the molecular mechanism that enables a CtBP monomer and dimer to act as a transcriptional activator and repressor, respectively. Although thorough clarification of these issues requires a huge amount of work, some additional data would be required to reach the standard of the EMBO Journal.

Specific comments:

1. In Figure 3, the authors claim that ectopic expression of either CtBPWT or CtBPMono in wing imaginal discs enhances Dll-LacZ expression. However, the enhancement of Dll-LacZ expression (Figure 3D and 3G) appears to be subtle and insignificant compared to control larva (Figure 3A). More convincing data with some quantitative analysis would strengthen the authors' conclusion.

2. Fig. 2A and C appear to contradict Fig. 4C and D. Fig 2A and B also contradict Fig. 3A and D. The authors need to explain these contradictions.

1st Revision - authors' response 01 February 2011

Authors' response to the referee's comments

Summary

We thank the referees for their supportive comments about our work and their constructive criticisms designed to improve the manuscript. Before addressing each referee's comments point-by-point, we would like to summarize their comments more generally and provide a short description of the major additions that are found in the revised manuscript.

Referee 1 made some thoughtful comments concerning the equilibrium between CtBP monomers and dimers in living cells. Although we demonstrate that mutant forms of CtBP that cannot dimerize can activate Wg signaling readouts, so can wild-type CtBP, which is competent for oligomerization. One key question is whether expression of wild-type CtBP causes an increase in CtBP dimers and monomers, or just oligomeric forms of CtBP. We have conducted co-IP experiments with differently tagged versions of CtBP and Acidic and Basic forms of CtBP that cannot homo-dimerize. This data is found in Figure 7 of the revised manuscript and demonstrates that even when one form of CtBP is present in 10-fold excess, significant levels of the other form remain in a non-CtBP dimeric form. This data supports a model where CtBP exists in both forms in living cells.

Another major concern raised by Referee 1 and 3 was the lack of mechanistic insight into how CtBP promotes activation of Wg signaling. In Figure 4C of the revised manuscript, we present data showing that a Gal4-Pygo fusion protein, which can efficiently activate a UAS-luciferase reporter in cultured cells, requires endogenous CtBP for this activity. Previous work from our lab (Fang et al 2006), showed that CtBP interacts with the N-terminal transactivation domain of Armadillo (Fang et al 2006). Our new results fit nicely with this knowledge, because it is the N-terminal transactivation domain of Armadillo that recruits the Legless/Pygopus complex to Wg targets (Kramps et al 2002). Given that Gal4-Pygo activates transcription independently of Arm and Legless (Stadeli and Basler, 2005), this data indicates that CtBP likely acts downstream of Pygo to activate Wg targets. This new result also fits with our data in Figure 2D-G, where monomeric CtBP can rescue the wing notch phenotype caused by overexpression of Pygo. Overexpression of Pygo blocks Wg signaling in several contexts possibly by disrupting the stoichimetry of a Legless-Pygo-SOMETHING ELSE complex (Parker et al 2002). We now propose that one "something else" is monomeric CtBP. Interestingly, wild-type CtBP cannot rescue the wing notch phenotype caused by Pygo, though this is difficult to interpret, since WT CtBP by itself promotes wing notching (Table I).

In response to referee's comments concerning the immunostaining data in Figure 3A, D & G, we have used image analysis software to quantify the signal strength, to better support the conclusions that we draw from this data (i.e., that both wild-type and monomeric CtBP can activate Dll-lacZ expression in wing imaginal discs).

Once again we thank the referee's for their comments and hope the experiments outlined above (also included in the point-by-point responses listed below) and our other responses to the referee's concerns will help convince everyone of the suitability of the revised manuscript for publication in EMBO Journal.

Point-by-point responses to Referee #1's comments:

1. Monomer/dimer distribution in cells

The authors show that dimerization is necessary for the repression activities of CtBP. They are careful not to make similar statements for the monomer form since they have shown only that it is sufficient for participation in activation; they have not shown monomers are required (i.e. that dimers don't activate). Dimer-competent wildtype CtBP can rescue activation just as well as CtBP monomer mutant (Fig. 4C, D, and E). This implies that either monomers are not required (i.e. dimers can activate in some settings and repress in other contexts) or that a significant portion of CtBP wildtype exists as a monomer. Can the authors show the distribution of CtBP monomer/dimers in cells? It appears to this referee that free monomer might not be abundant since co-expression of the acid/basic monomers appear to stabilize the levels of each form (Fig.6C, lanes 1,3 ad Fig. 4E inset).

Author's comments: To address the referees concerns about the size of the pool of monomeric CtBP in Kc cells, we co-expressed V5 CtBP with increasing amounts of Flagged tagged CtBP and subjected the cell lysates to Flag co-IP. As shown in Figure 7, even with a ten-fold excess of Flag-CtBP, under conditions were nearly all of the Flag-CtBP is precipitated, there is a significant portion of V5-CtBP remaining in the supernatant (Figure 7A top panel, lane 4). A similar result was obtained when V5 $CtBP^{Acidic}$ and Flag $CtBP^{Basic}$ were used. Since $CtBP^{Acidic}$ cannot homo-dimerize (Figure 6B), these data suggest that there is a significant pool of monomeric CtBP in Kc cells, under the conditions where our functional assays were performed.

The referee also correctly points out that our data allow us to say that CtBP monomers are competent for mediating the activation activity of CtBP. To make the stronger conclusion that CtBP monomers are required for activation, we would need a CtBP variant that can only exist as a dimer. We have attempted to prepare this type of CtBP, but making the dimer interface more hydrophobic (C134V, N138I, T143V, Y144F) resulted in a protein that had no activity in either repression or activation assays. We also attached two WT CtBP molecules with a 66 aa Gly-Ser linker (to provide enough linker for the antiparallel dimerization implied from the hCtBP1 structure). While the larger molecular weight linked protein was expressed at reasonable levels, there were shorter fragments including one of significant abundance at the size of monomeric CtBP, indicating an unacceptable degree of proteolysis of the linker.

Another idea suggested by the referee was to co-express CtBP acidic and CtBP basic, since they appear to stabilize each other (Figure 6C). This could create conditions where the expressed CtBP was in a predominately dimeric form. Co-expression of CtBP Acidic and Basic is able to

positively regulate Arm-mediated reporter expression (data not shown). But given the data shown in Figure 7, this is inconclusive, since there is significant non-dimeric CtBP under these conditions. These results suggest other mechanisms besides CtBP dimerization contribute to the stabilization effect of co-expressing CtBP acidic and basic.

2. Endogenous target genes

i. The authors use a modified fragment of the CG6234 gene for their reporter assays, including an 80bp internal deletion to increase responsiveness to Wg. Also, the region of the naked locus used for reporter activity is from an upstream region previously shown by this group not to be bound by endogenous CtBP or endogenous TCF (Fang et al. 2006). In light of these modifications and differences with past data, it is important to corroborate the findings from the reporter assays with chromatin immunoprecipitation at the corresponding endogenous sites.

Author's comments: The CG6234 WRE reporter is derived from the same region that is bound by endogenous CtBP and TCF (Fang et al 2006). The nkd UPE1 reporter is bound by endogenous CtBP (Figure S3) and endogenous TCF (Chang et al 2008).

ii. Likewise, RT-PCR for endogenous naked and CG6234 mRNA levels would test whether the reporter genes are faithfully mimicking effects on endogenous gene transcription.

Author's comments: We agree with the referee that the reporter genes used should faithfully recapitulate the regulation of the endogenous genes and we do provide support for this in Figures 4B and 5B, where the *CG6234* and *nkd UpE1* reporters are regulated by endogenous CtBP and TCF in a similar manner to that observed for *CG6234* and *nkd* mRNA (Fang et al. 2006). This point could be taken further by measuring these mRNAs under conditions where transgenic CtBP or various mutants are tested to see if they can rescue the loss of endogenous CtBP (as done with the reporter constructs in Figures 4D, 4E, 5C & 6D). However, the fact that CtBP RNAi affects >90% of the cultured cells, while our transfection efficiency is <10% makes this a technically challenging experiment. One approach is to establish stable transgenic expressing different CtBPs, but in our experience, this is not practical in Kc cells, where transgene expression levels vary widely in different stable cell lines.

3. Monomer recruitment versus monomer inactivity

i. The authors show that monomer CtBP does not support gene repression, but whether this inability is due to the fact that monomer cannot be recruited to regulatory sites, or whether it is recruited but not active is not addressed. The results shown in Figure 5C imply that monomer cannot be recruited for repression (monomer overexpression does not relieve repression under control RNAi conditions, meaning that it does not competitively displace endogenous dimers). A ChIP assay with tagged wildtype, tagged monomer, and/or tagged acidic/basic dimers would go far to test this part of the mechanism. That is, if monomer cannot be recruited to repression elements, then tagged acidic CtBP will only bind to regulatory sites in the presence of its basic heterodimer counterpart. In contrast, tagged acidic monomer and other tagged monomers should be capable of binding to sites for activation (CG6234) on their own.

Author's comments: We agree with the referee that these are interesting experiments. Unfortunately, we have not been able to obtain publication quality ChIP data for transfected CtBPs tagged with Flag.

ii. The authors show that acidic and basic heterodimers recapitulate CtBP repressor action on the Naked reporter construct. However, they do not test whether these monomer mutants work individually as activators for the CG6234 reporter. If acidic and basic monomers can activate CG6234 transcription, then co-expression of the mutants to reduce the monomer pool and form a large heterodimer pool, would test whether dimer formation interferes with activation of a CG6234 - a complementary experiment to the one above.

Author's comments: CtBP Acidic and Basic do activate an Arm-dependent readout, as does co-expression of these forms (data not shown). But given our results described in Figure 7 (that there is still high levels of non-dimeric CtBP under these conditions), we don't consider these results informative.

4. Minor comments

i. The authors state that DNA sequence context must influence CtBP action. However, CtBP monomers work to activate the artificial UAS-Luc reporter with Gal4-Arm fusion protein just fine. It does not appear that DNA sequence context plays a role in the activation part of CtBP

action.
Author's comments: We think that this result supports our context-dependent view of CtBP action on Arm-dependent transcription. In the simple UAS-luc reporter, the activating function is dominant, as is the case for CG6234-luc. In nkd UPE1-luc, there must be additional cis-acting elements that allow the repressing function of CtBP to occur. We have explained our view more clearly in the discussion (bottom of page 18) of the revised manuscript.

ii. The very nice phenotypes shown in Figure 2 shows that CtBP wildtype and CtBP monomers have different actions. On the other hand the Dll-LacZ reporter data in Figure 3 shows that they both activate the enhancer. Can the authors state, or otherwise discuss whether the majority of CtBP actions is dimer-mediated repression and that only a few genes (to date: CG6234, perhaps Dll) respond to CtBP activation?
Author's comments: The vast majority of CtBP's actions in transcriptional regulation are repressive. But in regard to Wg signaling readouts, it is more even, with WT and monomeric CtBP promoting UAS-luc, CG6234 and Dll-lacZ expression, while WT CtBP represses nkd expression and monomeric does not. 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* 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 signaling (Figure 2D-G), while WT CtBP promotes wing notching (Table I). We suspect that unlike UAS-luc, CG6234, Dll-lacZ and nkd, 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 WT CtBP expression and the activation with monomeric CtBP. We now discuss this in a paragraph on page 19/20 of the discussion.

Point-by-point responses to Referee #2's comments:

Introduction. P. 5. Is it known whether the repression role of CtBP requires enzymatic activity?
Author's comments: The NADH dehydrogenase activity of CtBP has been reported to be required for transcriptional repression in mammalian cell culture (Kumar et al 2002), but this conclusion has been challenged in several other papers (Grooteclaes et al 2003; Kuppuswamy et al 2008; Mani-Telang et al 2007; Phippen et al 2000; Sustias-Grau and Arnosti, 2004). There is a recent paper (Zhang and Arnosti 2011) showing that a catalytic site mutant of CtBP is not able to completely rescue the developmental phenotypes observed in CtBP mutants. In regard to Wg signaling, we have previously shown that several mutations in the active site of CtBP did not affect the ability of CtBP to activate Wg signaling. This information is now discussed in the introduction (bottom of page 5/top of page 6).

Fig. 3 would be clearer if both single channel images were shown in greyscale rather than color.
Author's comments: We have redone the figure as the reviewer suggests.

p. 9. The authors need to be a bit more detailed in their interpretation of the data in Figs 2 and 3. In the eye (Fig. 2), expression of CtBP wildtype and CtBP mono have opposite effects were as in the wing disc (Fig. 3) they have similar effects. While this is plausible, the authors should acknowledge this and provide a possible explanation.
Author's comments: This comment was also raised by referee 1 and 3, and has been addressed above. In short, we view the eye as a readout where the repressing or activating activity of CtBP can be observed, while the Dll-lacZ readout is only sensitive to the activating activity. A paragraph explaining this point has been added to the Discussion (page 19/20).

p. 11. The authors should mention in the first paragraph that their earlier data suggest CtBP can bind Arm, as this is necessary to understand how it can effect Gal4-Arm. It also would be worth mentioning this in the Introduction. Now, it only comes up late in the Discussion.*
Author's comments: This information is now included in the introduction (page 5, first paragraph).

Fig. 4B. It would be nice to also include a "blowup" of the 0 ng Arm data, to show whether the RNAi treatments have an effect on "basal levels"*
Author's comments: This is now included in Figure 4B of the revised manuscript. The depression

of CG6234 is about 2.6 fold in this experiment, which is at the high end of the range we have observed in several experiments.

Fig. 5. Why are the effects of CtBP WT and CtBP Mono opposite, instead of one simply being inactive in this assay?
Author's comments: We have observed an increase in nkd-UpE1 reporter activity when CtBP Mono is used to rescue CtBP RNAi several times, though it is not always observed (see Figure 6D). We suspect that CtBP Mono might have a weak dominant negative effect on CtBP repressing activity, which could account for our observations. A short comment mentioning this is now included in the results (bottom of page 13).

p. 13. I thought it was a bit of an overstatement to call the V5 tagged protein "HIGHLY unstable". Also, was the data in Fig. 6D generated with tagged forms of the protein?
Author's comments: "Highly" has been changed to "somewhat" in the results (page 15, first paragraph) to reflect the referee's valid point. The data obtained in Figure 6D was generated with Flag tagged forms of the protein and this is now more clearly pointed out in the Figure legend.

Point-by-point responses to Referee #3's comments:

The experiments described are straightforward and well-designed. The data are sound and appear to support the authors' conclusions. The results highlight the importance of the oligomeric state of CtBP in the Wg target-specific transcriptional regulation, and may be of interest to the readership of the EMBO Journal. However, my major concerns are: 1) upstream signaling that switches between monomeric and homodimeric CtBP…
Author's comments: To address the question of whether CtBP oligomeric state is influenced by Wg signaling, the effect of a stabilized form of Arm* to influence the degree of CtBP self-association was tested. We found no detectable change in the ability of Flag CtBP to pull down V5 CtBP plus or minus Wg signaling. This data is now provided in Supplemental Figure 6 and is now more clearly mentioned in the Discussion (page 23).

2) the molecular mechanism that enables a CtBP monomer and dimer to act as a transcriptional activator and repressor, respectively. Although thorough clarification of these issues requires a huge amount of work, some additional data would be required to reach the standard of the EMBO Journal.
Author's comments: To address the mechanism of CtBP's ability to promote activation of Wg signaling, we examined the connection of CtBP and Pygo in more detail. Pygo is required for Wg signaling (Parker et al 2002; Kramps et al 2002; Thompson et al 2002; Belenkaya et al 2002) and is thought to be the fourth protein recruited to form a quanterary complex (TCF-Arm-Legless-Pygo) on Wg response elements (Kramp et al 2002). Pygo is sufficient for transcriptional activation, as evidence by the ability of a Gal4-Pygo protein to activate a UAS-luc reporter (Stadeli and Basler, 2005). We now show that endogenous CtBP is required for the Gal4-Pygo to full activate this reporter (Figure 4C).

This data fit with two other pieces of evidence from our lab, one published and one included in this manuscript. Previously, we had shown that CtBP interacts with the N-terminal transcription activation domain of Armadillo (Fang et al 2006), which is the domain that binds to Legless-Pygo (Kramps et al 2002). In addition, we have shown that Pygo overexpression reduces Wg signaling, likely by disrupting the stiochiometry of a Legless-Pygo-UNKNOWN FACTOR(s). Our data suggests that CtBP is one of these unknown factors. This is supported by our data showing that monomeric CtBP can rescue the wing notch phenotype caused by Pygo overexpression (Figure 2D-G). We think it interesting that only monomeric CtBP can rescue the pygo wing notch phenotype, though this complicated by the fact that wild type CtBP promotes wing notches.

These results are now referred to in the abstract, introduction, results and discussion, and add to the mechanistic understanding of how CtBP acts in promoting Wg signaling.

Specific comments:

1. In Figure 3, the authors claim that ectopic expression of either CtBPWT or CtBPMono in wing imaginal discs enhances Dll-LacZ expression. However, the enhancement of Dll-LacZ expression (Figure 3D and 3G) appears to be subtle and insignificant compared to control larva (Figure 3A).

More convincing data with some quantitative analysis would strengthen the authors' conclusion.
Author's comments: We employed image analysis software to quantify the strength of the Dll-lacZ signal. As shown in Supplemental Figure 1, both WT CtBP and monomeric CtBP activate Dll-lacZ significantly higher than controls.

2. Fig. 2A and C appear to contradict Fig. 4C and D. Fig 2A and B also contradict Fig. 3A and D. The authors need to explain these contradictions.
Author's comments: This comment was also raised by referees 2 and 3, and has been addressed above. In short, we view the eye as a readout where the repressing or activating activity of CtBP can be observed, while the Dll-lacZ and CG6234-luc readouts are only sensitive to the activating activity. A paragraph explaining this point has been added to the Discussion (page 19/20).

2nd Editorial Decision 22 February 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74885R. It has now been seen again by referees 1 and 3, whose comments are appended below. As you will see, both referees are satisfied with your revision and are now fully supportive of publication. Referee 1 just has a couple of minor comments that I would first ask you to address in the text.

I also have a few points from the editorial side that need to be dealt with before we can accept the paper:
- Please can you include Author Contributions and Conflict of Interest statements (below the acknowledgments)?
- The resolution of the Westerns in Figure 1B seems to be rather low, and the figure looks a little pixelated. Please could you replace these panels with higher resolution images?
- In Figure 4D, the anti-tubulin control blot is off-centre, so that half the EV lane band is missing. Can you correct this?

Once we have the final version of the manuscript, with these last few changes, we should then be able to accept your study for publication without further delay.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This revised manuscript by Bhambhani et al., presents additional data on the oligomeric status of CtBP in cultured Drosophila cells, as well as new data suggesting an intriguing link to Pygopus, a co-activating factor for beta-catenin. Overall the revised manuscript is improved, with important new insights into CtBP, it's spectrum of activating and repressing activities and its intersection with the Wingless pathway. There are only two minor comments that are listed here:

Minor Comments:

1. Given that new data is presented for CtBP and Pygo, it would be good for the authors to discuss whether Pygopus activity is differentially required/involved in naked or CG6234 activation by Wingless - or not. This is not to say that new experiments should be performed, just that if the data is already known (published) - this should be highlighted.

2. The new sentence ending the Abstract is abrupt and the names "Pygo" and "Wg" are not defined.

Referee #3 (Remarks to the Author):

The manuscript has been adequately revised and is now suitable for publication.

2nd Revision - authors' response 09 March 2011

This cover letter accompanies our revised manuscript, in which we addressed the two specific comments of reviewer 2. Specifically, the end of the abstract has been reworded to improve the flow and better explain the relevance of the Pygopus-CtBP functional interaction. In addition, the reviewer asked about whether Pygopus activity is differentially required for naked and CG6234 activation by Wg signaling. We now mention in the discussion (bottom of page 21) our unpublished results that Pygopus is required for activation of both genes in Kc cells. This is not surprising, since pygopus mutants show a general loss of Wg signaling throughout fly development. These data suggest that there must be other gene-specific factors working with CtBP to explain its specific requirement for CG6234 activation (as opposed to naked where CtBP is not required). This is pointed out in the same paragraph in the discussion.

In addition, we have included improved versions of Figure 1 and 4 and the Author Contributions and Conflict of Interest statements below the acknowledgements. We hope that you will now find the manuscript acceptable for publication in the EMBO J and we are delighted at that possibility.

