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Modulation of Tcf3 repressor complex composition regulates cdx4 expression in zebrafish

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Transaction Report:

(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 03 January 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Firstly, please let me apologise again for the delay in getting back to you with a decision: unfortunately we did not receive all the referee reports before the Christmas break, and I have only just now returned to the office. However, we do now have a complete set of referees' comments, which are enclosed below.

As you will see, all three referees express interest in your identification of E4f1 as a regulator of Tcf3-dependent transcriptional repressor, but there are a number of major concerns with the study that would need to be addressed before we can consider publication in the EMBO Journal. Their reports are explicit and therefore I don't need to go into detail here, but I would in particular draw your attention to the comments of referee 3 regarding the need for better controls of the ChIP data, as well as further analysis of endogenous factors in zebrafish (as opposed to in cell culture).

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 of all three reviewers. 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

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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):

This manuscript is devoted to the regulation of the Cdx4 gene and its role in tail formation in zebrafish embryos. Experimental data suggest that Tcf3 in complex with corepressor proteins including Lnx2b keeps Cdx4 inactive, whereas E4f1 derepresses the Cdx4 gene. A Cdx4 reporter that includes TCF-binding sites in the 5' upstream regulatory region has been shown to mimic endogenous gene expression and respond to both the modulation of E4f1 levels and the Wnt pathway. These observations are supported by the analysis of tail development and the expression of Cdx4 and the Cdx4 target genes Hoxa9a and Gata1 in vivo. The ChIP data carried out on transfected HEK293 cells are consistent with a novel molecular mechanism, in which E4f1 decreases the binding of HDAC1 and Groucho/TLE repressors to the Cdx4 promoter without affecting Tcf3 binding. Most of the data are of high quality and appropriate for publication. To help improve the paper further, I recommend that the authors address the comments below.

1. Fig. S1B needs controls, in which the pull downs are done in the absence of E4f1.

2. page 7. Based on the analysis of Cdx4 MO effects, the authors propose that Cdx4 is a major target of Tcf3 repression that is involved in tail development, based on the rescue of Gata1 expression. It is surprising that they did not look at Hoxa9a that is more relevant for tail formation. Moreover, other Wnt target genes (e. g., Meis or Vent) are known to play a role in caudal development, besides Cdx4. The authors may wish to clarify relative contributions of these genes to the same developmental process, downstream of Wnt signals.

3. E4F1 does not bind to the Cdx4 promoter, but decreases the binding of exogenous corepressors HDAC1 and TLE3 to the Cdx4 promoter in transfected HEK 293 cells. The study would greatly benefit from demonstrating this result for endogenous proteins in zebrafish embryos.

4. Haremaki et al (Development, 2003,130:4907-17) reported that the functional TCF-binding sites are restricted mostly to the intronic region. How do results of the present manuscript compare with that study?

5. Fig. 1E. The effect of E4F1 MO alone is not presented here. If there is no effect, please state so.

6. Previous publications of the same group reported that Lnx2b influences organizer genes, which could have a secondary effect on tail development. Do the authors exclude this mechanism from consideration? A newly proposed mechanism is partly based on the experiments with a different Lnx2b MO, which has no effects on DV axis. These differences for two different MOs indicate potential lack of specificity and need to be discussed.

7. Fig. 2T, U. Cdx4 MO presumably has an effect by decreasing Cdx4 protein levels. Since E4f1 RNA stimulates Cdx4 expression, it would be expected to counteract the knockdown phenotype by increasing Cdx4 protein levels. The situation would be different in a genetic mutant that is a Cdx4 null. If these statements are correct, why does not E4f1 rescue Cdx4 morphants?

Referee #2 (Remarks to the Author):

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In this manuscript, Ro and Dawid present novel data elucidating a molecular mechanism regulating the expression of cdx4, a key regulator of caudal development in zebrafish embryos. This data clearly demonstrate novel interactions between the E4f1 transcription factor and Lnx-2b E3-ubiquitin ligase with the Tcf3 repressor protein and how the dynamics of these interactions may form a regulatory module governing cdx4 expression. Overall, the results are clearly presented and represent important findings that further the understanding of the caudal development program. However I do have a few points of concern that should be addressed before publication:

- The authors go to considerable lengths to show that a cdx4-luc reporter construct is transcriptionally activated by E4f1 in vitro and in vivo however E4f1 gain-of-function assays in vivo have no effect on endogenous cdx4 expression. Can the authors please elaborate more on this discrepancy?
- In the E4f1 rescue assays vs. the gain-of-function assays the authors indicate that they used either human or zebrafish E4f1 mRNA for these experiments. Is the stability of these mRNA's, injected at equivalent levels, comparable? This might explain why one mRNA efficient rescues the morphant phenotype but ectopic expression of the other homolog has no obvious effects.
- Results (page5): The rescue experiment is described as controlling for off-target toxic effects. This should probably read "non-specific effects" since non-specific effects are not necessarily toxic.
- Athough the E3 ligase activity of Lnx-2b was demonstrated not to play role in these studies, the authors may want to comment on whether the so-called atypical ubiquitin ligase activity of E4f1 plays a role in regulating the stability of components of the repressor complex.
- In results section titled, "E4f1 together with Wnt regulates cdx4 expression" and in supplementary information Figure 5, it is not clear/obvious what the parenthetically listed percentages actually represent?
- Supplementary information Fig 3: The authors state that e4f1 is uniformly expressed throughout all stages assayed. However, β-actin levels appear to be saturated making it difficult to determine the relative input influences on detected e4f1 levels.

Referee #3 (Remarks to the Author):

This manuscript is describes the role of E4f1 in regulating zebrafish tail development, cdx regulation and TCF3 function. Depletion of E4f1 causes a reduction in cdx4 expression and reduced tail elongation. This defect is enhanced by reduction in canonical Wnt signaling. In addition, the authors provide evidence that Wnt signaling regulates cdx4 expression through TCF3, and that Wnt signaling acts to relieve TCF3 repression of cdx4 transcription. The authors then connect E4f1 and TCF3 by demonstrating that E4f1 can inhibit the association of TCF3 with TLE co-repressors and HDACs. In addition, they provide evidence that Lnx-2b can antagonize this function of E4f1.

I think this manuscript provides new information about the relationships between Wnt signaling and Cdx genes in regulating the A/P axis in a vertebrate developmental system. In addition, it provides evidence for a new regulator (E4f1) in regulating TCF3 transcriptional activity by inhibiting co-repressor interaction with TCF3. While this mechanism is supported by strong data (e.g., Fig. 6C), the ChIP data is not adequately controlled and the authors make an assumption that what is true in HEK293 cells will be true in the zebrafish embryo. My comments are directed at strengthening these areas of the manuscript.

Major Comments/Concerns

1) The ChIP data in Fig. 4C uses IgG as a negative control. I think this is not sufficient. ChIP is a PCR-based assayed and false positive signals are a big concern. More primers sets around the cdx4 locus would provide additional confidence that there is an enrichment of TCF3 at the regulatory region. The best negative control is to show that the TCF3 mutant/morphant combination used in the manuscript has a significant reduction in TCF3 ChIP signal. Ideally, this data should be acquired with qPCR, so that readers can judge the data more critically than the qualitative presentations of DNA gels.

2) The authors would like the reader to believe that the putative TCF sites they have identified in the cdx4 regulatory region are functional. But there is no data to support this. They show that TCF3 is a

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repressor of cdx4 mRNA, which may or may not occur through the regulatory region they describe in the report. The functionality of the putative TCF sites can be tested by site-directed mutagenesis, easily done in HEK293 cells but more importantly in a transgenic zebrafish reporter.

3) A key argument in the manuscript is that Ef41 acts through TCF3 to regulate cdx4 expression. But the authors conclude this after Fig. 5 based solely on genetic interactions (top of page 11). This indicates a bias that should be removed from the manuscript. I don't see how the developmental genetics presented can distinguish between E4f1 acting through TCF3 or acting in parallel with TCF3 to regulate cdx4 expression. Fig. 3G also goes too far in pushing this one possibility for E4f1 action.

4) The data from Fig. 6E to Fig. 7 forms the basis for the mechanistic conclusions the authors make about how E4f1 works. Fig. 6E shows an impressive increase in TCF3-TLE or TCF3-HDAC interaction when E4f1 is depleted by siRNA. It would strengthen the paper considerably is E4f1 knockdown could be tied to regulation of the cdx4-luc reporter. If the model is true, then one would predict that beta-catenin would be less likely to counteract TCF3 repression of cdx4-luc expression.

5) I am suspicious of the sequential ChiP data shown in Fig. 7A. Getting reliable ChIP data on transfected reporters is very difficult, so I would need to see more controls (e.g., reduced binding when the TCF sites are destroyed) to believe what is essentially a negative result (that E4f1 overexpresssion does not effect TCF3 binding to the cdx4 regulatory region).

6) Fig. 7B needs qPCR to be convincing and should be combined with E4f1 siRNA to test the authors' favorite model more thoroughly.

1st Revision - authors' response 18 April 2011

Response to Reviewers

Referee #1:

This manuscript is devoted to the regulation of the Cdx4 gene and its role in tail formation in zebrafish embryos. Experimental data suggest that Tcf3 in complex with corepressor proteins including Lnx2b keeps Cdx4 inactive, whereas E4f1 derepresses the Cdx4 gene. A Cdx4 reporter that includes TCF-binding sites in the 5′ upstream regulatory region has been shown to mimic endogenous gene expression and respond to both the modulation of E4f1 levels and the Wnt pathway. These observations are supported by the analysis of tail development and the expression of Cdx4 and the Cdx4 target genes Hoxa9a and Gata1 in vivo. The ChIP data carried out on transfected HEK293 cells are consistent with a novel molecular mechanism, in which E4f1 decreases the binding of HDAC1 and Groucho/TLE repressors to the Cdx4 promoter without affecting Tcf3 binding. Most of the data are of high quality and appropriate for publication. To help improve the paper further, I recommend that the authors address the comments below.

1. Fig. S1B needs controls, in which the pull downs are done in the absence of E4f1.
→ We replaced the figure after adding a lane without E4f1, as requested (Supplementary Information Fig. S1B).

2. page 7. Based on the analysis of Cdx4 MO effects, the authors propose that Cdx4 is a major target of Tcf3 repression that is involved in tail development, based on the rescue of Gata1 expression. It is surprising that they did not look at Hoxa9a that is more relevant for tail formation. Moreover, other Wnt target genes (e. g., Meis or Vent) are known to play a role in caudal development, besides Cdx4. The authors may wish to clarify relative contributions of these genes to the same developmental process, downstream of Wnt signals.
→ *Cdx4* is known as master regulator of caudal development in zebrafish, and therefore we focused on this gene. In addition, in view of the fact that there are at least 47 *hox* genes in seven clusters in zebrafish, study of the upstream gene *cdx4* seems more practical. However, we report the response of *hoxa9a* to Wnt and E4f1 manipulations in Fig. 2. As regards other targets, we have now analyzed *axin2*, *lef1* and *tbx6* expression levels by RT-qPCR, now shown in Supplementary Information (Fig. S4D). These genes were affected by E4f1 knock-down in a modest but significant manner. We

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chose these genes instead of *meis* and *vent* for the following reasons. Meis is reported as a factor primarily functioning in establishing brain anterior-posterior patterning rather than tail development, and *vent* expression is largely dependent upon Bmp signaling rather than Wnt signaling in early development.

3. E4F1 does not bind to the Cdx4 promoter, but decreases the binding of exogenous corepressors HDAC1 and TLE3 to the Cdx4 promoter in transfected HEK 293 cells. The study would greatly benefit from demonstrating this result for endogenous proteins in zebrafish embryos.
→ Since the lack of available antibodies which can detect endogenous expression of Hdac1 and Groucho in zebrafish, we instead used cultured human cell system to verify the E4F1 dependent depression mechanism of cdx4. In this experiment we used siRNA against endogenously expressed E4F1, and ChIP with anti-HDAC1 and anti-TLE1/2/3/4 antibodies to precipitate endogenously expressed HDAC1 and TLE (Figure 7C).

4. Haremaki et al (Development, 2003,130:4907-17) reported that the functional TCF-binding sites are restricted mostly to the intronic region. How do results of the present manuscript compare with that study?
→ We added new data that show that LEF/TCF binding elements located in the *cdx4* 5' flanking region respond to Tcf3, whereas such elements in the 1st intron are important for the expression of *cdx4* but not for repression by Tcf3(Figure 4J and Supplementary Information Fig. S9). We believe that these data are compatible with the results of Haremaki et al.

5. Fig. 1E. The effect of E4F1 MO alone is not presented here. If there is no effect, please state so.
→ A photo of an E4f1 morphant is shown in Figure 1B.

6. Previous publications of the same group reported that Lnx2b influences organizer genes, which could have a secondary effect on tail development. Do the authors exclude this mechanism from consideration? A newly proposed mechanism is partly based on the experiments with a different Lnx2b MO, which has no effects on DV axis. These differences for two different MOs indicate potential lack of specificity and need to be discussed.
→ The different response to the two MOs is not a result of lacking specificity but a reflection of the biological context. Lnx2b mRNA is stored in the maternal pool, and protein translated from this pool is responsible for regulating the DV axis in the early embryo. The effect seen after inhibiting the translation of this maternal RNA by a translation-blocking MO cannot be seen when using a splice-blocking MO as the maternal RNA is already spliced. The spMO starts to affect development during gastrulation when maternal mRNA has decayed and newly synthesized zygotic mRNA takes over; this new mRNA is blocked from maturing by the spMO.

7. Fig. 2T, U. Cdx4 MO presumably has an effect by decreasing Cdx4 protein levels. Since E4f1 RNA stimulates Cdx4 expression, it would be expected to counteract the knockdown phenotype by increasing Cdx4 protein levels. The situation would be different in a genetic mutant that is a Cdx4 null. If these statements are correct, why does not E4f1 rescue Cdx4 morphants?
→ We believe that the cdx4 MO prevents translation of *cdx4* mRNA even when mRNA synthesis is stimulated by E4f1. This interpretation is the basis of placing *cdx4* downstream of E4f1 in the regulatory hierarchy of caudal body formation.

Referee #2:

In this manuscript, Ro and Dawid present novel data elucidating a molecular mechanism regulating the expression of cdx4, a key regulator of caudal development in zebrafish embryos. This data clearly demonstrate novel interactions between the E4f1 transcription factor and Lnx-2b E3-ubiquitin ligase with the Tcf3 repressor protein and how the dynamics of these interactions may form a regulatory module governing cdx4 expression. Overall, the results are clearly presented and represent important findings that further the understanding of the caudal development program. However I do have a few points of concern that should be addressed before publication:

- The authors go to considerable lengths to show that a cdx4-luc reporter construct is transcriptionally activated by E4f1 in vitro and in vivo however E4f1 gain-of-function assays in vivo

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have no effect on endogenous cdx4 expression. Can the authors please elaborate more on this discrepancy?
→ We believe that the reason why overexpression of E4f1 does not influence the endogenous expression level of *cdx4* is that E4f1 is strongly expressed both maternally and zygotically, probably reaching a saturating level. Other caudal genes (*axin2, lef1, tbx6*) which are also reported as Wnt targets, likewise did not substantially respond to *e4f1* mRNA injection. In contrast, similar to the reduction of *cdx4* expression, *axin2*, *lef1* and *tbx6* expression levels were compromised in *e4f1* morphants (Supplementary Information Fig. S4).

- *In the E4f1 rescue assays vs. the gain-of-function assays the authors indicate that they used either human or zebrafish E4f1 mRNA for these experiments. Is the stability of these mRNA's, injected at equivalent levels, comparable? This might explain why one mRNA efficiently rescues the morphant phenotype but ectopic expression of the other homolog has no obvious effects.*
→ Since the human *E4F1* mRNA does not contain the site targeted by *e4f1* MO, we used human *E4F1* mRNA to rescue the *e4f1* morphants. Furthermore this rescue experiment implies that the developmental role of E4F1 is evolutionally conserved from fish to human. In addition, similar to overexpression of zebrafish *e4f1* (100 pg of mRNA), injection of same amount of human *E4F1* mRNA did not generate any discernable morphological defects, presumably for the reasons outlined above. Thus we have no reason to suspect stability differences between the human and zebrafish E4f1 RNAs or proteins.

- *Results (page5): The rescue experiment is described as controlling for off-target toxic effects. This should probably read "non-specific effects" since non-specific effects are not necessarily toxic.*
→ Thank you for this comment. We edited our manuscripts by following the reviewer's comment, replacing 'off-target toxic effects' by 'non-specific effects'.

- *Although the E3 ligase activity of Lnx-2b was demonstrated not to play role in these studies, the authors may want to comment on whether the so-called atypical ubiquitin ligase activity of E4f1 plays a role in regulating the stability of components of the repressor complex.*
→ We could not detect any appreciable modulation of HDAC1 and Tcf3 protein levels by changing E4F1 expression levels (Figure 6A and 6E). Thus we are confident that E4f1 does not affect the stability of HDAC1 or Tcf3 in our system.

- *In results section titled, "E4f1 together with Wnt regulates cdx4 expression" and in supplementary information Figure 5, it is not clear/obvious what the parenthetically listed percentages actually represent?*
→ The numbers in parenthesis stand for the number of embryos analyzed; we added this explanation in the manuscript (Supplementary Figure legend S5).

Supplementary information Fig 3: The authors state that e4f1 is uniformly expressed throughout all stages assayed. However, b-actin levels appear to be saturated making it difficult to determine the relative input influences on detected e4f1 levels.
→ Thanks for the comment. We replaced the old figure with a repeat experiment, avoiding saturation of actin.

Referee #3:

This manuscript is describes the role of E4f1 in regulating zebrafish tail development, cdx regulation and TCF3 function. Depletion of E4f1 causes a reduction in cdx4 expression and reduced tail elongation. This defect is enhanced by reduction in canonical Wnt signaling. In addition, the authors provide evidence that Wnt signaling regulates cdx4 expression through TCF3, and that Wnt signaling acts to relieve TCF3 repression of cdx4 transcription. The authors then connect E4f1 and TCF3 by demonstrating that E4f1 can inhibit the association of TCF3 with TLE co-repressors and HDACs. In addition, they provide evidence that Lnx-2b can antagonize this function of E4f1.

I think this manuscript provides new information about the relationships between Wnt signaling and Cdx genes in regulating the A/P axis in a vertebrate developmental system. In addition, it provides evidence for a new regulator (E4f1) in regulating TCF3 transcriptional activity by inhibiting co-

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repressor interaction with TCF3. While this mechanism is supported by strong data (e.g., Fig. 6C), the ChIP data is not adequately controlled and the authors make an assumption that what is true in HEK293 cells will be true in the zebrafish embryo. My comments are directed at strengthening these areas of the manuscript.

Major Comments/Concerns

1) The ChIP data in Fig. 4C uses IgG as a negative control. I think this is not sufficient. ChIP is a PCR-based assayed and false positive signals are a big concern. More primers sets around the cdx4 locus would provide additional confidence that there is an enrichment of TCF3 at the regulatory region. The best negative control is to show that the TCF3 mutant/morphant combination used in the manuscript has a significant reduction in TCF3 ChIP signal. Ideally, this data should be acquired with qPCR, so that readers can judge the data more critically than the qualitative presentations of DNA gels.
→ We designed additional primer sets to test the binding of Tcf3a to the LEF/TCF consensus sites in the *cdx4* regulatory region(Supplementary Information Fig. S8). Furthermore we found several additional LEF/TCF binding elements in the 1st intron, and the binding of Tcf3 to these elements was also tested (Figure 4A-C). Following the reviewer's helpful comment, we carried out in vivo ChIP by comparison WT with Tcf3a mutant embryos (*MZhdl*) using qPCR, as requested (Figure 4D).

2) The authors would like the reader to believe that the putative TCF sites they have identified in the cdx4 regulatory region are functional. But there is no data to support this. They show that TCF3 is a repressor of cdx4 mRNA, which may or may not occur through the regulatory region they describe in the report. The functionality of the putative TCF sites can be tested by site-directed mutagenesis, easily done in HEK293 cells but more importantly in a transgenic zebrafish reporter.
→ Through mutagenesis of the putative LEF/TCF binding sites we found that the LEF/TCF binding elements lodged in 5' flanking region of *cdx4* are responsive to Tcf3 repression whereas the consensus elements in the 1st intron have a role in *cdx4* expression but not Tcf responsiveness. We also found that removal of all putative LEF/TCF consensus sites by a combination of deletion and point mutation rendered the reporter inactive in the zebrafish embryo (Figure 4J and Supplementary Information Fig.S9).

3) A key argument in the manuscript is that Ef41 acts through TCF3 to regulate cdx4 expression. But the authors conclude this after Fig. 5 based solely on genetic interactions (top of page 11). This indicates a bias that should be removed from the manuscript. I don't see how the developmental genetics presented can distinguish between E4f1 acting through TCF3 or acting in parallel with TCF3 to regulate cdx4 expression. Fig. 3G also goes too far in pushing this one possibility for E4f1 action.
→ Following the reviewer's comments, we removed the interpretation that E4f1 acts through Tcf3. We present data showing that Tcf3-depleted embryos fail to respond to E4f1 manipulations without bias, as requested. In addition we deleted the original Figure 3G, as requested.

4) The data from Fig. 6E to Fig. 7 forms the basis for the mechanistic conclusions the authors make about how E4f1 works. Fig. 6E shows an impressive increase in TCF3-TLE or TCF3-HDAC interaction when E4f1 is depleted by siRNA. It would strengthen the paper considerably is E4f1 knockdown could be tied to regulation of the cdx4-luc reporter. If the model is true, then one would predict that beta-catenin would be less likely to counteract TCF3 repression of cdx4-luc expression.
→ The reduction of cdx4-luc activity after depleting E4f1 using a specific morpholino (MO) is shown in Figure 3E; these results were consistent in three independent experiments. We further show in Fig. 4E that β-catenin can derepress but not substantially activate cdx4-luc in the presence of Tcf3.

5) I am suspicious of the sequential ChiP data shown in Fig. 7A. Getting reliable ChIP data on transfected reporters is very difficult, so I would need to see more controls (e.g., reduced binding when the TCF sites are destroyed) to believe what is essentially a negative result (that E4f1 overexpression does not effect TCF3 binding to the cdx4 regulatory region).
→ We have compared wild type and mutant reporter constructs to test the binding of Tcf3 to the *cdx4* regulatory region in the presence and absence of E4f1 (Fig. 7A). This approach is more direct than the approach in our original manuscript, and uses the mutant reporter construct to test binding

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site specificity, as requested by the reviewer. The results show that E4f1 does not interfere with binding of Tcf3 to cognate sites in the upstream region and 1st intron of WT *cdx4*, while the mutant reporter construct does not support substantial enrichment of immunoprecipitated consensus regions.

6) Fig. 7B needs qPCR to be convincing and should be combined with E4f1 siRNA to test the authors' favorite model more thoroughly.
→ Following the comments of the reviewer, we carried out qPCR in this experiment. The new results strongly support our conclusions, as shown in the new Figure 7B. We also carried out ChIP-qPCR assays with anti-HDAC1 and anti-TLE antibodies after depleting E4F1 using siRNA, as requested by the reviewer. The new results (Figure 7C) show that HDAC1/TLE binding to Tcf3 consensus sites increases strongly after depletion of E4F1, supporting the model in Figure 7D.

2nd Editorial Decision 16 May 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76589R. It has now been seen again by referees 1 and 3, whose comments are enclosed below. As you will see, both referees find the study to be significantly improved and are now fully supportive of publication. I am therefore pleased to be able to tell you that we can accept your manuscript to be published in EMBOJ.

I do, however, just have a couple of issues from the editorial side first.
- In figure 6, the blots all appear very highly contrasted, and I am concerned as to how well these reflect the original data. I would therefore ask you to provide more representative panels here. For our records, I also need to ask you to send me the original scans of the blots used to assemble this figure.
- Please can you ensure that experimental replicate number and statistical tests used are clearly stated in all figure legends where relevant - I noticed that these are missing in some places?
- At the moment, you have two supplementary information files; please can you combine these into a single PDF, so that the entire SI is in one file?
- We require Author Contributions and Conflict of Interest statements for all accepted manuscripts: could you please include these below the Acknowledgements section?

Once we have a final version of the manuscript with these various changes, we should then be able to accept your paper without further delay.

Many thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!

Best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors made an impressive effort to address the reviewers' comments. The revised manuscript has been significantly improved and is acceptable for publication.

Referee #3 (Remarks to the Author):

I am satisfied with the extensive revisions that the authors have made in response to my comments. I think this is a very well done and significant manuscript.

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2nd Revision - authors' response 17 May 2011

Thank you for your message and for accepting our paper for publication in EMBO Journal. We appreciate your and the reviewers' help in improving the manuscript.

We upload a revised text and Supplementary Information as requested, and I send the original scans of the films for Figure 6 as attachments to this message as there is no suitable place to include them in the uploaded files. We attach the original JPEG files of the scanned films, and a PowerPoint assembly of all the scans for overview. We hope you will find them satisfactory.

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