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Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/β-Catenin signaling

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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 15 April 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are enclosed below. As you will see, all three referees recognise the potential importance of your work, but raise major concerns that would currently preclude publication in the EMBO Journal. In particular, all three find that the mechanism by which Oct-3/4 induces beta-catenin degradation has not been well elucidated, and that a significantly more in-depth analysis would be required. In addition, all three question the physiological significance of your results, both in the context of ES cells and in Xenopus development. This is particularly important given that your results seem to contradict previous publications that suggested cooperation between Oct-3/4 and the Wnt pathway in the maintenance of ES cell pluripotency.

It is unclear to us whether you will be able to resolve these concerns, and the work required would certainly go beyond the scope of a normal revision. Therefore, I am afraid I can not offer to consider a revised version of your manuscript. However, although we do not usually consider resubmissions, we do recognise the interest in your work. Therefore, we would be willing to consider a resubmission in this case, should you be able to significantly strengthen your study along the lines indicated by the referees - both in terms of the mechanistic insight and the physiological relevance. I should point out that this would then be treated as a new submission and would have to be considered again, in the light of any literature published in the interim period, and possibly also involving new referees.

I am sorry we can not be more positive at this stage, but I hope you find the referees' comments useful, and thank you in any case for the opportunity to consider your manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Abu-Remaileh et al. describes a direct interaction of oct-3/4 and b-catenin that can reduce b-catenin stability and downstream signaling in mouse ES cells and in early embryonic development of Xenopus. They suggest that this regulatory mechanism might restrict b-catenin signaling and help to control the balance between maintenance of pluripotency and differentiation. In addition, they describe an effect of this regulatory mechanism for the induction of EMT which might play a role in mesoderm induction and gastrulation.
While these results are interesting many important questions remain unresolved. The work therefore needs considerable revision prior to publication.

1) A very important question that remains unresolved: Why/How does binding of oct-3/4 to b-catenin induce degradation?
1a) Is oct-3/4 part of the degradation complex?
1b) Does oct-3/4 affect the composition/activity of the degradation complex and if so how?
1c) Since oct-3/4 is mainly nuclear and the degradation complex cytoplasmic: does the interaction between oct-3/4 and b-catenin occur in the nucleus or in the cytoplasm? Does it affect the localization of b-catenin?

2) Previous studies have come to different conclusions: a direct interaction between oct-3/4 and TCF3 has been reported previously and these results are questioned by the authors without showing own experimental evidence.
2a) Please include immunoprecipitation data to test for complexes between oct-3/4 and TCF3 in your system.
2b) Also interaction studies using only purified bacterial proteins need to be included in order to exclude indirect interactions via larger multi-protein complexes.
2c) In addition, previous experiments (Hochedlinger et al, Cell 2005;121:465) have overexpressed oct-3/4 in epithelial cells and have reported upregulation of b-catenin signaling instead of downregulation. Please repeat the interaction studies of oct-3/4 and b-catenin in keratinocytes to assess whether this interaction is cell type specific.

3) What is the in vivo relevance of this concept? The gene ablation of b-catenin affects embryonic axis formation and mesoderm induction which involves EMT. Therefore the observation that oct-3/4 repression induces EMT is of interest. However, the expression patterns in mouse do not fit to this idea. At E6.5, Oct-3/4 is expressed throughout the whole epiblast while Wnt signaling is restricted to the posterior site and the primitive streak area. One day later, oct-3/4 gets restricted to the primitive streak region, the area of highest Wnt signaling activity; this contradicts their concept. In addition, the embryonic phenotype of oct-3/4 ablation affects epiblast formation and shows no resemblance to the b-catenin KO or APC KO phenotypes.
3a) The authors need to provide in vivo evidence that their suggested mechanism is relevant for embryonic development. Does this fine-tuning of b-catenin signaling have a biological readout? Do subtle differences in b-catenin signaling strength indeed alter normal embryonic development and is this oct4 mediated?
3b) For the Xenopus experiments only the mouse gene is used to block Wnt8 induced secondary axis formation. This is very artificial as two Xenopus homologues (oct25 and oct60) exist. Please

repeat these experiments with the Xenopus genes to establish whether oct mediated repression of Wnt signaling plays a role in Xenopus development.
3c) Related to this, what is the expression pattern of oct25 and oct60 in relation to Wnt pathway targets such as siamois? Do these patterns support your concept?
3d) You have used morpholinos against oct25 and oct60. What is the embryonic phenotype of these morpholinos and how does that relate to effects on endogenous Wnt signaling activity?

4) The conservation/similarity of murine oct-3/4 and the Xenopus homologues is rather low in the N-terminus. Please show that the mechanism of b-catenin degradation is conserved and involves the N-terminus of the Xenopus proteins.

5) The DN-TCF3 block of differentiation induced by oct-3/4 withdrawal involves enhanced repression of TCF3 targets and is therefore not the appropriate experiment to prove a role of oct-3/4 mediated regulation of b-catenin. Please repeat this experiment using knock down of b-catenin instead.

Referee #2 (Remarks to the Author):

The authors investigate the interaction between b-catenin and oct3/4. They show that the two proteins directly interact, and that Oct-3/4 overexpression promotes β-catenin degradation. Experiments in ES cells indicate that downregulation of Oct-3/4 initiates, via enhanced Wnt transcription, cell motility. The authors suggest that the balance between β-catenin and Oct-3/4 is regulates stemness and differentiation.

There is already quite a bit of data showing an interaction between b-catenin and oct3/4 so the novelty of the present data is somewhat compromised (notably PMID 18467660 and 17196549). The most relevant new findings of the study are direct oct4 interaction and destabilization of b-catenin. The physiological relevance is less stringently analyzed since most of the data entail overexpression. The mechanism of oct4 mediated b-cat degradation remains unclear (enhanced Gsk3 access to b-cat and phosphorylation or the likes?). Furthermore the requirement for the described interaction in Xenopus remains unclear; the authors implicate EMT; is this relevant in Xenopus? In conclusion, the study reports on a new interaction of two very important regulators of cell differentiation and pluripotency and is therefore of great relevance. However, it falls somewhat short in describing the physiological relevance and mechanism.

Other points

1. Fig. 1B: unclear how experiment was done; is this cumulative luc activity from t0= or was each luc transfection done after the end of RA incubation?

2. p.12 The authors suggest that one main mechanism of oct4 action on ES cells is via Wnt/b-catenin (as opposed to a myriad of oct4 target genes). This would be quite spectacular. Could the authors build on the data and corroborate them further?

3. The ms is in parts poorly written, imprecise, wordy and inaccurate:

"Oct-3/4 specifically interacts with β-catenin and induces its degradation via the canonical Wnt signaling pathway." Awkward phrase; canonical Wnt signaling pathway is not degrading anything; proteaseome or lysosomes are.

"Consequently, Wnt signaling was suppressed in both early Xenopus embryos and embryonic stem (ES) cells." There is tons of literature about Wnt signaling in Xenopus and ES cells; how can it be suppressed?

“Oct-3/4... is a key regulator of pluripotency during the earliest stages of vertebrate development." The relevant literature is not quoted. How about Schöler et al. 1990 for starters?

The following phrase shows little concern for the reader:

"Using a cutoff value of a Benjamini-Hochberg adjusted p value of <0.05, 878 known genes (identified by UCSC Genome Database) from the Affymetrix Mouse Gene 1.0 ST Array were significantly different (by at least 2 fold) in dox-treated ZHBTc4 and ZHBTc4/DN2-5 cells;"

Referee #3 (Remarks to the Author):

In the presented manuscript the authors report a novel regulatory interaction between Oct-3/4 and β-catenin in embryonic stem cells. They confirm the previously shown physical interaction between both proteins by co-immunoprecipitation, both from 293T cells after over-expression and from ESCs after proteasome inhibition. In a number of in-vitro experiments and expression in Xenopus embryos the authors intend to show Oct-3/4 dependent degradation of β-catenin by the canonical destruction complex. Whilst they show stabilization of β-catenin in SW-480 cells and after proteasome inhibition (both with or without Oct-3/4 coexpression), they fail to present a direct link of the Oct-3/4/β-catenin dimer and the destruction complex, ubiquitinilation or proteasome, nor do they suggest a putative mechanism of the Oct-3/4 guided β-catenin degradation. For instance the sub-cellular location of such interaction remains unclear, Oct-3/4 being a nuclear protein, the canonical destruction complex exclusively localizing to the cytosol. Further clarification of the molecular mechanism of this process would significantly improve the impact of the presented work.

The authors conclude from their data that Oct-3/4 dependent degradation of β-catenin is required to establish a "certain level of nuclear β-catenin" which is "compatible with proliferation and pluripotency". In a previous publication (Takao et al. 2007; cited by the authors for demonstrating the interaction of Oct-3/4 and β-catenin), it has been shown, that β-catenin and Oct-3/4 cooperate in actively transcribing the pluripotency factor Nanog. Further in this study, stabilization of β-catenin results in prolonged maintenance of the undifferentiated state of ESCs after Lif withdrawal rather than in an EMT-like differentiation. Other reports (Sato et al. 2005; Silva et al. 2008) show that GSK3 inhibition (as the central component of the β-catenin degradation complex) is beneficial for ESC maintenance and even reprogramming of somatic cells to a pluripotent state.

The discrepancies between the new regulative mechanism presented in this manuscript and the previously published evidence is too large and not satisfyingly addressed nor discussed to recommend the work, as it is, for publication in the EMBO journal.

Finally all but a few experiments were done using highly artificial system of transient transfection in cells in which molecules of interest normally do not exist and the processes of interest do not occur. There is no evidence that Oct4 causes degradation of β-catenin in ES cells. Upon differentiation of ES cells level of Oct4 is going down and the level of β-catenin is going up but there is no evidence that these changes are causally related.

Specific comments:

1. In Fig 2A, no endogenous β-catenin was detected in the uninjected embryos, while in Fig 2B, there appeared to be a lot of endogenous β-catenin in the uninjected controls. Why was there a discrepancy between the two controls?

2. Fig 2C and Fig 2D, what were the amounts of Oct3/4 transfected? In Fig 2D, even at the maximal Oct3/4 amounts, there was still residual Flag-β-catenin, while in Fig 2C, no β-catenin was detectable. Was the amount of Oct3/4 transfected in Fig 2C much more than used in Fig 2D?

3. There were no descriptions of the gel shift in Fig 2G in the text. The band for Oct1 appeared to be non-specific.

4. The graph in Fig 3A lacked error bars - were replicate experiments not performed?

5. In Fig 3A, it is clear that β-catenin degradation occurs in the control cells without Oct4. This indicates Wnt signaling is absent in these cells and β-catenin is mostly present in the cytosolic compartment. It is, therefore, hard to reconcile how Oct4, a nuclear factor, can mediate the

proteolytic degradation of -catenin in the cytoplasm. What happens when Wnt signaling is induced in the Oct4 containing cells?

6. Fig. 3B. It is not good scientific practice to combine lanes from different gels/experiments to illustrate what should have been a single properly controlled experiment.

7. Page 8-9. I am not sure that these experiments prove that Oct 4 induces fl-catenin degradation via the canonical pathway. There is no evidence that there is any other pathway for β-catenin degradation except the canonical one involving destruction complex and proteosomal degradation. When that pathway is blocked β-catenin cannot be degraded regardless of what Oct 4 does.

8. Fig 4. The authors should perform a reciprocal IP for Oct3/4.

9. Depletion of Tcf3 has been shown to result in ESCs that are resistant to differentiation, due to de-repression of target genes Oct4 and Nanog (Pereira et al, 2006, Tam et al, 2008, Yi et al, 2008). The authors should demonstrate that the phenotypes of DN2-5 in Fig 5A-B were not caused by elevation of Nanog levels. The authors should also show that the DN2-5 Tcf3 mutant can still interact with co-repressors to repress the expression of Nanog.

10. Interpretation of Kemler et al., 2004 reference is erroneous (pages 4 and 16)

1st Resubmission 28 March 2010

It is our pleasure to submit this revision of our manuscript "Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/b-catenin signaling." As you will notice, we have addressed all of the main points raised by the reviewers by providing comprehensive new data both with regard to the mechanism by which Oct-3/4 induces b-catenin degradation (Fig. 3A, C-F and Supplementary Fig. 1 and 2A-C), as well as the functional significance of our novel results, both in the context of ES biology and in *Xenopus* development (Fig. 4E,F and Supplementary Fig. 4D,E, 5A-C). As a result of the new data we reorganized the figures, and the detailed conversion scheme of the old to new figures is depicted at the end of this letter. With these additions and corrections, we truly believe that the manuscript is much stronger and is appropriate for publication in the EMBO Journal.

Reviewer #1

1. This is an excellent and important question and we have invested a great deal of effort into deciphering the mechanism by which Oct-3/4 induces b-catenin degradation. We have greatly expanded experiments in order to respond to points 1a, b and c.

   1a- As requested by the reviewer we have now shown that the complex that contains Oct-3/4 and b-catenin also contains Axin1, which is part of the degradation complex (Fig. 3C and Supplementary Fig. 2A).

   1b- In order to investigate whether Oct-3/4 affects the composition/activity of the degradation complex, we analyzed the phosphorylation pattern of b-catenin in the presence or absence of Oct-3/4. To this end, we carried out three experiments. First, we showed that in MG-132-treated 293T cells the level of b-catenin phosphorylation of serine 33 and 37 (generated by GSK beta, a component of the degradation complex) increases in the presence of Oct-3/4 (Supplementary Fig. 2C). Second, we repeated the same experiment in RKO cells and obtained the same result (data not shown). Third and most relevant, we showed that in ES cells once expression of Oct-3/4 is down-modulated (due to dox treatment) phosphorylation of serine 33 and 37 decreases in the nucleus and not in the cytoplasm, indicating that GSK phosphorylation, that is a prerequisite for the proteasome-dependent degradation, occurs in the nucleus and in an Oct-3/4-dependent manner (Fig. 3F).

   1c- We agree with the reviewer that it is pertinent to know where in the cell the interaction between Oct-3/4 and b-catenin occurs. Thus, we isolated cytoplasmic and nuclear fractions and clearly showed that Oct-3/4-b-catenin interaction is detected in the nucleus (Fig. 3C,D and

Supplementary Fig. 2B). Moreover, we clearly showed that Oct-3/4 induces the degradation of nuclear b-catenin (Fig. 3E,F). Our results are in accordance with published data showing GSK3, Axin1, and the proteasome machinery in the nucleus (Brooks et al., 2000; Palmer et al., 1994; Palmer et al., 1996; Reits et al., 1997; Zhou et al., 2004).

In summary, we have unraveled a number of crucial mechanistic aspects of Oct-3/4-dependent b-catenin degradation. However, we are aware that additional experiments are needed in order to decipher the details of this degradation pathway, more so since there are a number of unsolved questions in this field. We have clearly stated this point in the Discussion section of our manuscript.

2. 2a- In no way do we question the previously published interaction between Oct-3/4 and Tcf3, and thus we have chosen not to repeat the published experiments. Oct-3/4 is known to interact with a growing list of proteins, such as Sox2 and Nanog, Tcf3 and b-catenin certainly belong to this list.

   2b- We also do not claim that the interaction between b-catenin and Oct-3/4 is a direct one, however, previously published pull-down experiments have already demonstrated that bacterially expressed GST-b-catenin (an activated mutant that is not degradable) interacts with Oct-3/4. We do not doubt these results either, and they are cited in our manuscript (Takao et al., 2007).

   2c- The reviewer is correct in citing Hochedlinger *et al.*'s experiments in which they have clearly shown that ectopically expressed Oct-3/4 elevated b-catenin levels (mostly in the intestine). In skin of adult mice they could not observe a consistent increase in b-catenin activity upon Oct-3/4 induction. They did see it in embryonic skin. These results led them to suggest that activation of Wnt/b-catenin cascade may be one of the key downstream events of Oct-3/4-induced progenitor-cell expansion. However, a subsequent paper from the same group has clearly shown that Oct-3/4 gene ablation (in several somatic tissues, including intestine, hair follicle, liver, etc.), revealed no abnormalities in regenerative capacity of somatic stem cells. Therefore it was concluded that Oct-3/4 does not play a crucial role in adult somatic cells including adult stem cells (Lengner et al., 2007). Thus, we feel that studying the interactions between Oct-3/4 and b-catenin in keratinocytes (even fetal) has no advantage over other somatic cells, nevertheless we cited their work in our manuscript.

3. 3a,b- The reviewer is correct in noting that experiments pertinent to the *in vivo* relevance of our novel findings are important. It was exactly for this reason that we turned to the *Xenopus* system, which is more amenable to this kind of study. As the reviewer noted in his/her comment, the situation in the mouse system is much too complicated, yielding phenotypes that are harder to characterize and reconcile, and are technically much more challenging.

   As requested by the reviewer we have now shown that Oct-25 and Oct-60 can effectively block the second axis induction by Xwnt-8, suggesting that POU-V mediated repression of Wnt signaling plays a role in *Xenopus* development (Fig. 4E). Moreover, we have carried out additional experiments showing that fine-tuning of b-catenin signaling by POU-V has a physiological significance in *Xenopus*. First, we showed that antisense morpholino oligo directed against Oct-25 and Oct-60 increased secondary axis formation induced by Wnt/b-catenin signaling (Fig. 4F). Second, we showed that there is a specific developmental window of time when knockdown of Oct-25 and Oct-60 led to elevated expression of b-catenin targets (Supplementary Fig. 4). These results may indicate that also during mouse embryogenesis Oct-3/4-induced b-catenin degradation occurs at a specific development window of time. Third, we showed that the increase in TOPFLASH expression in injected *Xenopus* embryos due to co-injection of morpholino oligos directed against Oct-25 and 60 was almost completely abolished by knocking-down b-catenin. All together these gain- and loss-of function experiments clearly attest to the physiological significance of our results in *Xenopus* embryos. We also added experiments that show the physiological significance in the context of ES cells (see below #5, as requested by this reviewer).

   3c,d- The expression pattern of Oct-25 and Oct-60 in relation to Wnt pathway targets, such as Siamois, supports our concept. POU-V factors are primarily localized in the animal half and their concentration decreases toward the vegetal pole. In contrast, fertilization results in accumulation of b-catenin at the dorsal side where it enhances transcription of factors, such as

Siamois, which dorsalize mesendodermal germ layers (Cao et al., 2007). These results fit well with previous results published from the same group showing that injection of embryos with antisense morpholino oligos to Oct-25 and Oct-60 results in elevated transcription of mesendodermal marker genes (Cao et al., 2006).

4. As described above, the *Xenopus* system served us mainly to study questions that are much harder to approach using the mammalian embryo. To study the mechanism by which Oct-3/4 induces b-catenin degradation, we mostly used mammalian somatic and embryonic cell culture systems. This is the reason we performed most of the degradation experiments in mammalian cells using the Oct-3/4 protein. However, the reviewer is correct in asking to connect between the phenotype we observe in the manipulated *Xenopus* embryos and the degradation process using *Xenopus* proteins. Therefore, we performed transfection experiments using Oct-25 and Oct-60 expression vectors into 293T cells. We were unable to show that Oct-60, either alone or together with Oct-25, is able to degrade b-catenin, most probably due the lack of *Xenopus* specific co-factors. As an alternative approach, we transfected cells with a very small amount of Oct-3/4 that on its own is unable to induce degradation, together with increasing amounts of Oct-25 and demonstrated that nuclear b-catenin levels were reduced by Oct-25 in a dose-dependent manner. These new data are now included in our revised manuscript (Supplementary Fig.1).
5. As requested by the reviewer, we have now repeated the experiment using shRNA construct to b-catenin. We showed that similarly to DN-Tcf3-expressing cells, migration of shb-catenin cells was almost completely inhibited. Moreover, since these clones still express b-catenin, it is clear that fine-tuning of b-catenin levels has a biological readout, strengthening the physiological significance of our results in the context of ES cells as well. These data are now noted in the paper (Supplementary Fig. 5A-C).

Reviewer #2

We have now carried out extensive studies to demonstrate the physiological relevance of our findings, mostly using loss-of-function (knockdown) strategies (rather than overexpression).

1- We have now shown that antisense morpholino oligos against Oct-25 and Oct-60 increased secondary axis formation induced by Wnt/b-catenin signaling (Fig. 4F).

2- We have demonstrated that the increase in TOPFLASH expression in injected *Xenopus* embryos due to co-injection of morpholinos against Oct-25 and 60 was almost completely abolished by knocking-down b-catenin (Supplementary Fig. 4E).

3- We have carried out experiments showing that Oct-26 and Oct-60 effectively block second axis induction by Xwnt-8 (Fig. 4E).

4- We have introduced shRNA to β-catenin into ES cells, in which Oct-3/4 expression can be down-modulated by dox treatment. We showed that similarly to DN-Tcf3, shβ-catenin dramatically affected cell migration.

When taken together, these experiments indicate that fine-tuning of b-catenin signaling by POU-V/Oct-3/4 has a physiological significance in *Xenopus* embryos and in the context of ES cells. These data are now all noted in the revised paper.

We have greatly expanded the biochemical experiments in order to decipher the mechanism by which Oct-3/4 induces β-catenin degradation.

1- We have now clearly shown that the interaction between β-catenin and Oct-3/4 occurs exclusively in the nucleus (Fig. 3D and Supplementary Fig. 2B).

2- We have demonstrated that Oct-3/4-induced β-catenin degradation takes place in the nucleus only (Fig. 3E,F).

3- We have now shown that the complex containing Oct-3/4 and β-catenin also contains Axin1, which is part of the degradation complex (Fig. 3C and Supplementary Fig. 2A).

4- We have analyzed the phosphorylation pattern of β-catenin in the presence or absence of Oct-3/4. To this end we carried out three experiments.

4a. We have shown that in 293T cells the level of β-catenin phosphorylation of serine 33 and 37 (generated by GSK beta, a component of the degradation complex) increases in the presence of Oct-3/4 (Supplementary Fig. 2C).

4b. We have repeated the same experiment in RKO cells and obtained the same result (data not shown).

4c. We have shown that in ES cells once expression of Oct-3/4 is down-modulated (due to dox treatment), phosphorylation of serine 33 and 37 decreases in the nucleus, and not in the cytoplasm, indicating that GSK phosphorylation occurs in the nucleus, and in an Oct-3/4-dependent manner (Fig. 3F).

All together these results demonstrate that Oct-3/4 affects the composition/activity of the degradation complex, and we described these results in our revised manuscript.

In summary, we have unraveled a number of crucial mechanistic aspects of Oct-3/4-dependent β-catenin degradation. However, we are aware that additional experiments are needed in order to decipher the details of this degradation pathway, more so since the there are a number of unsolved questions in this field. We have clearly stated this point in the Discussion section of our manuscript.

Other points

1. Fig. 1B-Luciferase reporter transfections were done at time 0 and extracts were taken for analysis 16, 30 and 52 hours following dox treatment. It was made clear in the text.
2. It was not our intention to suggest that Oct-3/4 regulates cell fate decisions mainly through its ability to induce β-catenin degradation, as opposed to transcriptional activation of hundreds of target genes. However, since the Wnt/β-catenin signaling pathway is involved in virtually every aspect of embryonic development, we suggest that a cross talk, like the one we described between Oct-3/4 and β-catenin, is of an utmost importance for early embryonic decisions.
3. Our manuscript underwent professional editing. Thank you for pointing it out to us.

Reviewer #3

As requested by the reviewer, we have greatly expanded the biochemical experiments in order to clarify the link among Oct-3/4, β-catenin and the degradation complex.

1- We have now clearly shown that the interaction between β-catenin and Oct-3/4 occurs exclusively in the nucleus (Fig. 3D and Supplementary Fig. 2B).

2- We have demonstrated that Oct-3/4-induced β-catenin degradation takes place in the nucleus only (Fig. 3E,F).

3- We have now shown that the complex containing Oct-3/4 and β-catenin also contains Axin1, which is part of the degradation complex (Fig. 3C and Supplementary Fig. 2A).

4- We have analyzed the phosphorylation pattern of β-catenin in the presence or absence of Oct-3/4. To this end we carried out three experiments.

4a. We have shown that in 293T cells the level of β-catenin phosphorylation of serine 33 and 37 (generated by GSK beta, a component of the degradation complex) increases in the presence of Oct-3/4 (Supplementary Fig. 2C).

4b. We have repeated the same experiment in RKO cells and obtained the same result (data not shown).

4c. We have shown that in ES cells once expression of Oct-3/4 is down-modulated (due to dox treatment), phosphorylation of serine 33 and 37 decreases in the nucleus, and not in the cytoplasm, indicating that GSK phosphorylation occurs in the nucleus, and in an Oct-3/4-dependent manner (Fig. 3F).

All together these results demonstrate that Oct-3/4 affects the composition/activity of the degradation complex, and we described these results in our revised manuscript.

In summary, we have unraveled a number of crucial mechanistic aspects of Oct-3/4-dependent β-catenin degradation. However, we are aware that additional experiments are needed in order to decipher the details of this degradation pathway, more so since the there are a number of unsolved questions in this field. We have clearly stated this point in the Discussion section of our manuscript.

It should be noted that previously published papers have demonstrated that proteasomes are abundant in both cytosol and nucleus (Brooks et al., 2000; Palmer et al., 1994; Palmer et al., 1996; Reits et al., 1997; Zhou et al., 2004). It has been shown that endogenous p53 undergoes degradation both in the nucleus and in the cytoplasm (Joseph et al., 2003), and more recently published papers show that the proteolytic activity of the proteasome in the nucleus is required for transcription (reviewed (Collins and Tansey, 2006; Kodadek et al., 2006). These (and additional published) studies clearly show that ubiquitin-mediated proteasomal degradation occurs in the nucleus, and thus strongly support our findings demonstrating that Oct-3/4 induced β-catenin degradation in the nucleus. Our data does not exclude the possibility that enhanced nuclear cytoplasmic shuttling contributes to the decrease in nuclear β-catenin, although the lack of any increase in cytoplasmic β-catenin suggests that an effect of shuttling is small. We have clearly noted this in our Discussion section.

The reviewer is correct in pointing out that there are several papers showing that β-catenin signaling is beneficial for ES and iPS establishment. In fact, there are contradicting reports as to β-catenin role in ES cell biology. It seems that β-catenin has a dual role in ES biology, i.e., maintaining stem cell identity and directing ES cell differentiation. Several studies have shown that activation of the Wnt pathway can cause ES cells to remain pluripotent under conditions that induce differentiation (Hao et al., 2006; Kielman et al., 2002; Miyabayashi et al., 2007; Ogawa et al., 2006; Sato et al., 2004a; Singla et al., 2006; Takao et al., 2007). For example, it has been shown that the ability and sensitivity of ES cells to differentiate is inhibited by increased doses of β-catenin, which are the result of specific mutations in the *APC* gene (Kielman et al., 2002). In addition, GSK-3-specific pharmacological inhibitors were shown to maintain the undifferentiated phenotype of ES cells (Sato et al., 2004b), and to assist in reprogramming of somatic cells (Silva et al., 2008). More recently, several Wnts have been shown to enhance the ability of LIF to maintain self-renewal of ES cells (Hao et al., 2006; Ogawa et al., 2006) and, consistently, overexpression of the Wnt antagonist Sfrp2 was found to stimulate the production of neuronal progenitors (Aubert et al., 2002).

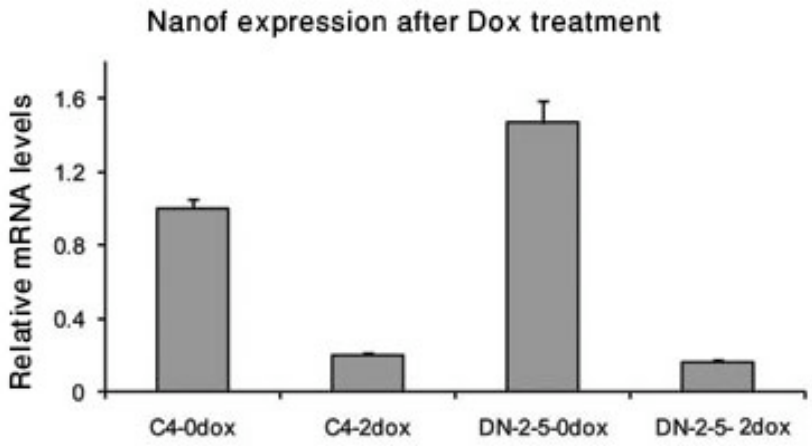
However, other studies have shown that the Wnt pathway has an important role in directing differentiation of ES cells (Lindsley et al., 2006; Otero et al., 2004). It was shown that β-catenin/Tcf activity is low in undifferentiated cells and increases at the onset of both human and murine ES differentiation, and that this signaling pathway is critical for neural differentiation of ES cells. It was also reported that the addition of Wnt3a stimulates not only ES cell proliferation, but also differentiation (Dravid et al., 2005), and that inhibition of endogenous Wnt signaling abrogates the generation of ES-cell derived mesoderm (Lindsley et al., 2006). Accordingly, canonical Wnt signaling is required for the expression of genes associated with primitive streak, endoderm and mesoderm (Lindsley et al., 2006).

Interestingly, as it turns out, a number of genome-wide analysis studies support our working findings by showing that in ES cells no co-localization of β-catenin-Tcf3 was observed on target genes, and immuno-staining revealed that β-catenin was largely restricted to the cytoplasm of ES cells, with very little nuclear localization (Tam et al., 2008). When the same group examined several loci at greater resolution they showed that whereas co-repressors, such as TLE2 and CtBP, bind several target genes together with Tcf3, β-catenin was not found to be bound. Importantly, β-catenin did not appear to be localized in the nuclei of ICM cells as well. Accordingly, no evidence for active Wnt/β-catenin signaling during pre-implantation development was found (Kemler et al., 2004). In addition, the same group has shown that stabilization of β-catenin (using a β-catenin mutant) leads to premature EMT in the epiblast. Furthermore, it is interesting to note that the intracellular expression pattern of Oct-3/4 and β-catenin in ES cells supports our model; i.e., the level of Oct-3/4 is high in the nucleus while the level of β-catenin is low. When taken together, all these studies suggest that Wnt/β-catenin regulation is a key event in stem cell fate determination (self-renewal or differentiation) that most likely depends on extrinsic signals (such as FGF, BMP, LIF) and intrinsic cell factors (such as Oct-3/4, our results). In our revised manuscript, we now provide a better discussion of this complex issue.

We appreciate the reviewer request to study the process not only in 293T cells but also in ES. These are technically very challenging experiments. We succeeded to show that in ES cells Oct-3/4 causes degradation of β-catenin, in the nucleus and that Oct-3/4 induced β-catenin phosphorylation of serine 32 and 37. We have now added these data to the revised manuscript (Figure 3F).

Specific comments:

1. There is no discrepancy between the two controls in figure 2A and 2B (new figure 4A and 4B). In (old) figure 2A we transfected a β-catenin construct and thus exposure time was very short, and as a consequence we could not detect the endogenous β-catenin protein. In (old) figure 2B we only transfected the Oct-3/4 expression construct and examined the endogenous β-catenin (as indicated in the figure). Exposure time was much longer.
2. Since figure 2D essentially includes figure 2C we decided to present only figure 2D (new figure 1C). The amount of transfected Oct-3/4 was noted in the text.
3. We have now added a description of the gel shift analysis in the text. The Oct-1 band is not a nonspecific band. Oct-1 is ubiquitously expressed and it binds to the OCTA oligonucleotide. Therefore, we expect it to appear in each transfected lane, albeit stronger in the Oct-1 transfected cells, which it did.
4. We have now noted the number of repeated experiments and included the error bars for data presented in figure 3A (new figure 2A).
5. We have now clearly shown that Oct-3/4-induced β-catenin degradation occurs in the nucleus, and these data are included in the revised manuscript. The reviewer is correct in noting that inducing b-catenin signaling in ES cells is of importance. However, we have chosen not to perform this experiment since it was carried out previously, as discussed above (p. 9,10).
6. We agree with the reviewer, therefore, most of our autoradiograms are from a single gel. Only in few figures we had to combine lanes, due to repeated technical problems, but even there we always had the +/- Oct-3/4 on the same gel with the same exposure time (old Fig. 3B, new Fig. 2B).
7. The reviewer is correct. However, we have employed several experimental approaches to show that Oct-3/4-induced β-catenin degradation, only one was by blocking the proteasomal degradation machinery.
8. The old Fig. 4 was exchanged with the new Fig. 3 that contains a number of reciprocal experiments, some shown in the figure itself and others in Supplementary Fig. 2.
9. Indeed, we studied Nanog expression in DN2-5 and found it to be very low as shown in the figure below. This is most probably due to the presence of DN-Tcf3 which can still interact with its co-repressors.
10. The interpretation of Kemler et al has been corrected.



Figures

New Figures	Old Figures
1A	1A
1B	1B
1C	2D
1D	2E
1E	2F
1F	2G
1G	3D
2A	3A
2B	3B
2C	3C
3A	New
3B	4B
3C	New
3D	New
3E	New
3F	New
4A	2A
4B	2B
4C	1D
4D	1C
4E	New
4F	New
5 (A-D)	5 (A-D)
6 (A-D)	6 (A-D)
Supplementary Figures	
1	New
2 (A-C)	New
3A	1A
3B	1B

4A	1C
4B	1D
4C	1E
4D	New
4E	New
5 (A-C)	New

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Tam, W.L., Lim, C.Y., Han, J., Zhang, J., Ang, Y.S., Ng, H.H., Yang, H. and Lim, B. (2008) T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. *Stem Cells*, 26, 2019-2031.

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2nd Editorial Decision 15 April 2010

Many thanks for submitting the new version of your manuscript EMBOJ-2010-74322. It has now been seen again by all three of the original referees, whose comments are appended below. As you will see, referees 2 and 3 are now supportive of publication. Referee 1, however, has serious remaining concerns, primarily concerned with how your results relate to previously published reports. There are a number of issues here. Firstly, the referee questions the novelty of your results in the light of Cao et al, which already showed that Oct3/4 negatively regulates Wnt signalling in the Xenopus embryo. I do recognise that you propose a different mechanism here, and that you also provide evidence for the functional relevance of this regulation in ES cells, and am therefore inclined to set aside this concern. Secondly, it has previously been proposed (Takao et al) that beta-catenin and Oct3/4 positively cooperate to regulate self-renewal of ES cells. While we recognise that you do provide good evidence for a negative relationship, it would be critical that this contradiction is adequately discussed.

Thirdly, and most importantly, this referee still has strong concerns as to your model for how Oct3/4

regulates beta-catenin, and does not find your evidence for Oct3/4-mediated nuclear degradation of beta-catenin to be sufficiently convincing. This is especially important in the light of the multiple studies showing that nuclear export is important for beta-catenin degradation. Having looked at the data myself, and discussed it in detail with my colleagues, I have to say that we share this referee's concern. Particularly given the somewhat controversial nature of your proposal, and that this is a central aspect of your study, we therefore feel that it would be essential that you provide stronger evidence for your model before we could consider publication in the EMBO Journal.

Since your manuscript is a resubmission, rather than a revision, there is still the opportunity for you to undertake a further round of revision to address this critical point. I would therefore like to invite you to revise your manuscript by providing new experiments with further evidence to support your model that Oct3/4 directly promotes nuclear degradation of beta-catenin (as well as to exclude other, more indirect models as suggested by the referee). Currently, the best evidence - looking at the effects of removing endogenous Oct4 - is presented in Figure 3F. However, it is not clear to me that the levels of cytoplasmic beta-catenin are in fact unchanged under your Oct4 knockout conditions. It would therefore be essential that this experiment be replicated, and the data quantified. In this context, I also have to raise an editorial concern with the data presented in Figure 3. The blots shown in all panels have been extensively cropped, and there are multiple bands half-visible in several of the panels. Therefore, when you submit your revised manuscript, I would ask you to also include, as supplementary information, the original scans of all blots for the data presented in this figure. I should stress that a revised version of your manuscript will have to be seen again by Referee 1, and eventual acceptance of your study would depend on your satisfying this referee that you have sufficient evidence to support the claim for Oct3/4-mediated nuclear degradation of beta-catenin.

Please do not hesitate to get in touch should you have any questions or comments regarding the revision of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Overall, the manuscript has improved considerably. However, the ES cell part contradicts earlier reports which describe an important function of b-catenin/Oct4 interaction for ES cell self-renewal (Takao et al, 2007). Moreover, it is still not clear whether they basically describe the same phenomenon as Cao et al. EMBO 2007 or whether they have identified a new mechanism. Many of the central experiments have been published previously by Cao et al. in 2006 and 2007, and this new work by Abu-Remaileh et al. does not expand the biological relevance of this Oct4 / TCF / b-catenin crosstalk to other models. So the only novel aspect of this new work would be the Oct4 induced degradation of b-catenin. Whilst they have somewhat expanded this part, these new data raise many additional questions as they contradict published results in the field. While a function of the proteasome in the nucleus has been described previously, this is not the case for nuclear b-catenin degradation. Previous studies have shown that nuclear export of b-catenin by APC or axin is a prerequisite for b-catenin degradation and for controlling Wnt signalling (Aberle et al., 1997; Cong and Varmus, 2004; Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2003). Mutants of APC or axin which affect their nuclear import or export were shown to be sufficient for preventing b-catenin degradation and thereby activating Wnt signalling. These results are challenged by the current manuscript. It is so far not clear whether this loss of nuclear b-catenin is mediated via nuclear degradation, shuttling of the protein between nucleus and cytoplasm (which would better conform to previous results) or even indirectly via the previously shown interaction of Oct4 and TCF3 (which may displace b-catenin from the nucleus and thereby induce degradation).

Referee #2 (Remarks to the Author):

The authors have addressed my points adequately and the ms can be published

Referee #3 (Remarks to the Author):

The authors responded to significant majority of comments and criticism and added substantial amount of new experimental evidence to support their conclusions. The revised manuscript can be accepted for publication.

1st Revision - Authors' Response 11 July 2010

It is our pleasure to resubmit our revised manuscript "Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/β-catenin signaling." We were happy to read that Reviewers 2 and 3 recommended publication of our manuscript. As you will notice, we have addressed all of the points raised by Reviewer 1 and by you.

We addressed the contradiction between our manuscript and the data published by Takao et al., showing that β-catenin up-regulates Nanog expression through interaction with Oct-3/4 and Tcf3/β-catenin in the Discussion (p. 22). In this section we noted that the relationship between Oct- 3/4 and Tcf3/β-catenin pathway is complex. It was previously shown that Tcf3, Nanog and Oct-3/4 all co-occupied the promoters of one another. Moreover, it was shown that in ES cells Tcf3 functions mainly as a repressor via interactions with TLE2 and/or CtBP co-repressors. Repression of Oct-3/4 and presumably Nanog can be relieved by stimulation of Wnt signaling through the addition of Wnt3a (Tam et al., 2008). Since activation of the Wnt signaling results in accumulation of nuclear β-catenin, it is highly possible that over expression of non-degradable β-catenin mutant (Takao et al., 2007), that cannot be degraded by Oct-3/4, results in over accumulation of nuclear β-catenin. This in turn results in activation of Nanog and Oct-3/4, most probably through displacement of the Tcf3/co-respressor complex, which brings about maintenance of ES self renewal in this particular context. However, it should be noted that genome wide analysis did not reveal any colocalization of β-catenin with Oct-3/4 in ES cells, and that in these cells β-catenin is largely localized in the cytoplasm, casting doubts on the physiological significance of Takao et al.'s data.

More importantly, we provide convincing new experiments using ES cells, supporting our model that Oct-3/4 promotes nuclear β-catenin degradation, and exclude other models (suggested by Reviewer 1), such as Oct-3/4- dependent shuttling of β-catenin between nucleus and cytoplasm, or Oct-3/4- 2 dependent displacement of β-catenin from the nucleus due to Oct-3/4-Tcf3 interactions. These data are presented in Fig. 3G, Supplementary Fig. 2C and Discussion.

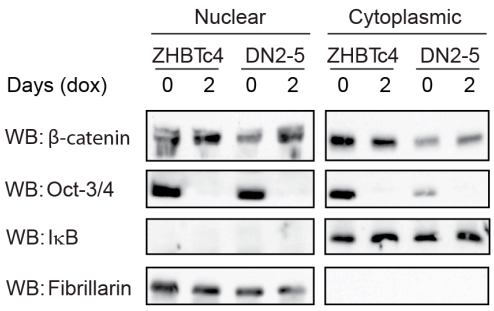
As requested, we repeated the experiments presented in Fig. 3F three times; one presented in Fig. 3F, the second one in Supplementary Fig. 2C and the third is presented below. The data from these experiments was quantified and presented in Supplementary Fig. 2C. All experiments clearly show that removal of endogenous Oct-3/4 resulted in up regulation of endogenous β- catenin in the nuclear fraction only. In fact, as is clearly presented in the quantitation of the cytosolic data, the level of β-catenin was not affected by down-modulating Oct-3/4 expression.

As requested, we have attached all the original scans of the blots for the data presented in Fig. 3, as well as the blots presented in Supplementary Fig. 2 which are related to results depicted in Fig. 3.

These new experiments and information should go a long way towards clarifying elements of the paper that were not clear, and we truly believe that our manuscript is appropriate for publication in the EMBO Journal.

References

Takao, Y., Yokota, T. and Koide, H. (2007) Beta-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem Biophys Res Commun*, **353**, 699-705.
Tam, W.L., Lim, C.Y., Han, J., Zhang, J., Ang, Y.S., Ng, H.H., Yang, H. and Lim, B. (2008) T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. *Stem Cells*, **26**, 2019-2031.



Response to Reviewer #1

The reviewer is correct in noting that the regulation of b-catenin activity is thought to occur also via nuclear-cytoplasmic shuttling. Indeed, it was shown that both axin and APC function as molecular chaperones for b-catenin, thereby enriching b-catenin in the cytoplasm. However, a more recent study shows that axin and APC mainly regulate b-catenin sub-cellular localization by retaining it in the compartment in which they are localized, rather than by active transport into the cytoplasm (Krieghoff et al., 2006). In fact, they showed that axin and APC are much less mobile in the cytoplasm than b-catenin, probably because of their association with fixed structures such as cytoskeleton, and they substantially reduce b-catenin mobility in the cytoplasm. Thus, nuclear-cytoplasmic distribution of b-catenin is still a matter of debate.

Nevertheless, we agree with Reviewer 1 that it its is important to investigate whether preventing export of nuclear b-catenin to the cytoplasm in ES cells would interfere with the ability of Oct-3/4 to induce b-catenin degradation in the nucleus. We treated ES cells with LMB, a highly specific inhibitor of the CRAM1-dependent export pathway, and protein levels of b-catenin were measured in nuclear and cytosolic fractions. Results from these experiments clearly showed that LMB treatment did not alter nuclear β-catenin levels, indicating that Oct-3/4 does not reduce nuclear β-catenin through facilitating its export to the cytoplasm. These new data are now presented in Fig. 3G of the revised manuscript.

We also investigated whether the interaction between Oct-3/4 and Tcf3 in ES cells results in displacement of β-catenin from the nucleus and thereby induce its degradation. To investigate this issue we made use of ES cells expressing a dominant-negative Tcf3 construct lacking the N-terminal region, required for interaction with b-catenin. These cells were treated with dox and β-catenin levels were assessed in the nuclear and cytosolic fractions. Results from these experiments clearly show that down-modulating endogenous Oct-3/4 expression levels by dox treatment results in up-regulation of endogenous b-catenin expression in the nuclear fractions of both wild type and dominant negative expressing ES cells. These results indicate that in dominant-negative Tcf3 expressing ES cells b-catenin accumulates in the nucleus to the same degree as in wt cells, although it mostly does not bind Tcf3. Thus, it is highly unlikely that Oct-3/4 ability to bind Tcf3 results in b-catenin displacement from the nucleus. These new data are presented in Supplementary Fig. 2C of the revised manuscript.

These new results (presented in figures described above and in the Results and Discussion sections), together with the rest of our experiments strongly indicate that Oct-3/4 reduces b-catenin protein levels through its nuclear degradation, rather than shuttling or displacement mechanisms.

Reference

Krieghoff, E., Behrens, J. and Mayr, B. (2006) Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. *J Cell Sci*, **119**, 1453-1463.

3rd Editorial Decision 22 July 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-74322R to the EMBO Journal. I had asked referee 1 to look at the revised version, but unfortunately he/she is not available to do so at this time. However, having gone through it carefully myself, and discussed it with my colleagues, I am pleased to be able to tell you that we can accept your manuscript for publication, without the need to seek additional external input, or for further revision.

Just a couple of things I need to make you aware of. It is EMBOJ policy to make the original scans of blots available to the reader if they have been requested by the editorial office. I would therefore propose to make a separate supplementary file containing the scans you sent me - I hope this is okay for you. Also, I just want to check that you are happy for us to publish the Review Process File, including the figures you incorporated into your point-by-point response and cover letter during the previous rounds of review.

You should receive the formal acceptance message from our editorial office shortly; if you could just get back to me to confirm that you are okay with the points above, that would be great.

Yours sincerely,

Editor
The EMBO Journal

