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Smg6/Est1 licenses embryonic stem cell differentiation via nonsense-mediated mRNA decay

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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.)

Editor: Anne Nielsen

1st Editorial Decision 16 October 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the slight delay in communicating our decision to you. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express great interest in the findings reported in your manuscript and support publication of an adequately revised version. However, they do raise a number of major and minor concerns that will have to be addressed before such a revision can be submitted. I will not go through all the individual points raised, but it is clear that two aspects of the study need to be developed further:
You will have to provide more direct evidence for the regulation of c-myc by NMD (ref#1 and #2) and expand the characterization of the SMG6-depleted ESCs to rule out transformation rather than loss of differentiation capacity (ref #3).

In addition, I would encourage you to focus your efforts on the following points:

-> supplement the analysis with more endogenous NMD targets (ref #1 point 2)

-> provide further insight on the role for Smg6 in cell proliferation (ref#1 point 5, ref #3 points 1, 4, 9)
-> data-mine for known c-myc targets and correlate with expression data from Smg6-depleted cells (ref #1 point 8); this should also help you address some of the concerns from ref #3 regarding RNA seq data and c-myc expression
-> conduct the isoform analysis suggested by ref #2 to address a possible NMD-escape mechanism by c-myc
-> it would also be helpful if some additional IF data for differentiation markers could be included (ref #1 point 6, ref #3 point 3) but this will not be an absolute requirement from out side

The referees also bring up a number of minor concerns with quantification, controls and data descriptions that should be amended/discussed as far as possible.

Given 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, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

REFEREE COMMENTS

Referee #1:

Li et al. is an intriguing study providing strong evidence that a post-transcriptional pathway promotes both the differentiation and generation of pluripotent stem cells. This discovery fills an important niche, as the vast majority of work in the field of pluripotent cell differentiation/reprogramming has, instead, been focused on transcriptional pathways. The authors show several lines of evidence that the RNA degradation pathway, NMD, is responsible for these effects on pluripotent cells (e.g., by knocking down different NMD factors) and they elegantly rule out another possibility - that the telomerase-promoting activity of NMD factors is responsible. Importantly, they also identify a particular transcript - c-myc mRNA - that NMD downregulates to promote ES differentiation. Together, this study represents a major step forward towards understanding the underlying molecular mechanisms responsible for going to-and-from the pluripotent state.

While I have a lot of enthusiasm for this manuscript, there are some issues that must be dealt with, both major and minor.

Major Issues:

(1) c-myc is a NMD target. Since the authors are claiming that they have identified a NMD circuit involving c-myc mRNA, it is absolutely essential that they demonstrate that this transcript is indeed a NMD target. At the very least, the authors must (i) test whether c-myc mRNA is not only upregulated by SMG6 knockdown, but also in response to KD of 1 or more other NMD factors (since SMG6 has activities in addition to NMD), (ii) test whether c-myc mRNA is stabilized by SMG6 KD (since the hallmark of NMD is that it destabilizes its targets), and (iii) identify at least one feature in c-myc RNA that could be responsible for it being targeted by NMD pathway; e.g., an intron in the 3' UTR, a long 3' UTR, and an uORF are all known NMD-inducing features. In addition, it would be preferable (but not required) if the authors empirically tested whether this feature triggers NMD. It would also strengthen this manuscript if the authors tested whether an NMD-insensitive form of c-myc mRNA prevents mES cell differentiation. Of note, if the authors find that c-myc mRNA is NOT a direct NMD target, that is ok, but then they should investigate (at least to some degree) the underlying mechanism by which NMD indirectly regulates c-myc (e.g., is it transcriptionally or post-transcriptionally regulated by NMD?).

(2) Endogenous NMD targets. The NMD target transcripts the authors have chosen to measure NMD magnitude in response to loss of SMG6 are either also induced by stress (e.g., ATF4), which complicates interpretation of the results, or are non-coding RNAs (e.g., GAS5), which may be degraded by NMD in an unconventional way since they lack a long ORF. Therefore, the authors should also include well-characterized protein-coding NMD substrates mRNAs that are not linked with stress.

(3) Exogenous SMG6. The levels of exogenous SMG6 (in its various form) in the rescue experiments must be shown. It is important to know their level relative to endogenous SMG6 to fully interpret the experiments.

(4) Effect of loss of SMG6 in ESCs. On the bottom of pg. 5, it is indicated that loss of SMG6 has no effect on proliferation or morphology, but no quantification of passage numbers or proliferation rate is provided. Related to this, the authors claim that there are little or no telomeres in SMG6-mutant cells; this seems incompatible with survival, and thus should be addressed.

(5) NMD, c-myc, and proliferation. There is a vast literature showing that c-myc promotes proliferation and also some literature showing the same for NMD. Thus, it is surprising that the authors do not find that loss of the NMD factor, SMG6, has much of any effect on proliferation. The authors should address this. They should also address the fact that NMD opposes the expression of c-myc and thus one might expect that NMD and c-myc would serve opposite roles. In the case of embryoid bodies, they refer to Smg6-mutant cells proliferating more than control cells, but do not show the data (pg. 15). They should show the data and discuss it in light of the previously known roles of NMD and c-myc in proliferation. Likewise, they should also experimentally address (using FACS and/or EdU labeling) or at least discuss the role of NMD/c-myc in undifferentiated ES cells and MEFs.

(6) Fig. 1F. It would be preferable if lineage-specific markers were used to definitively assess the germ layer defects.

(7) Fig. 2A. The Smg6-null cells appear to be considerably smaller than the control cells. This should be noted, along with speculation as to why this might be so.

(8) c-myc targets. It would elevate this manuscript if the authors identified the complete set of candidate genes that NMD regulates through c-myc. This can easily be done by mining known c-myc target genes (defined from published databases) as to whether they correspond to transcripts dysregulated in the authors' Smg6-mutant RNA-seq datasets.

Minor Issues:

(1) Fig. 5B. It is critical that all functional categories that are statistically over-represented ($P<0.05$) are shown in this figure. The precise P values should also be indicated. Finally, the authors should provide not only the numerator (# proteins corresponding to mRNAs dysregulated) but also the denominator (the number of proteins in a given category).
(2) There are several over-statements in this manuscript, which need to be toned down. For example, in the Abstract, it says that lethality as a result of NMD factor loss is caused by defects in homeostasis. This is not necessarily true, so the word "indicates" is too strong.
(3) This manuscript needs major copy-editing. A few examples include: (i) Abstract: the first and second sentences have no transition; (ii) Abstract, last line, should be changed to something like: "...both the differentiation of stem cells and the reprogramming of differentiated cells into stem cells;" (iii) Pg. 4, first sentence of first full paragraph: "involved," not "involves;" and (iv) Pg. 13, near top: insert "other" between "knock down" and "NMD factors." This is only a partial list; the entire manuscript needs to be carefully scrutinized.
(4) Correct word usage. In addition to improvements in grammar and style, more appropriate word usage should be implemented. For example, on pg. 4, "...may exaggerate the complexity...in vivo" should be replaced with something like "...complicates the interpretation of phenotypic effects that result from their loss." On pg. 9, first sentence of the first full paragraph, the word "genes" should be replaced with "RNAs" or "transcripts," as NMD acts on RNAs, not genes (also see the same error in

the next paragraph). As another example, on pg. 11, RNA-seq analysis does not measure "transcriptional levels," it measures "RNA levels." Just below that, it states that "the global transcriptional profile did not change...," when in fact the RNA prolife (note, not the "transcriptional profile") DID change (>2000 genes changed in level!); phrasing needs to be altered to reflect a general similarity in RNA profile but statistically significant changes in ~10% of the transcriptome. The entire manuscript should be carefully screened for these kinds of errors.
(5) It is strongly suggested to mention that the Smg6-mutant ESCs and MEFs have a deficiency in NMD when these mutant cells are first discussed. Refer to data that will be presented later.
(6) The Discussion requires a more in-depth analysis of previously published studies showing a connection between NMD and proliferation/cancer; e.g., Wang et al. MCB '11 previously showed that c-myc inhibits NMD (which coupled with the data in the present paper, suggests a mutually enforcing feedback loop) and Liu et al. Nature Medicine '14 showed that inactivating mutations in the NMD gene, UPF1, are likely to cause a specific type of cancer.
(7) The authors should comment on whether or not it is known if KO or KD of telomerase factors besides NMD factors are required for ES cell differentiation.

Referee #2:

Li and colleagues have investigated here the effect of Smg6 ablation on embryonic cells' capacity to differentiate. Since smg6 nulls were embryonic lethal, they generated conditional Smg6 knockouts in mouse embryonic stem cells and fibroblasts, which both were viable and showed no detectable proliferation or morphological anormalities. However, through a set of well-controlled experiments the authors document compellingly that the Smg6 deletion blocks ESC differentiation in vitro and in vivo and that the MEFs are severely compromised in the de-differentiation to iPSCs. The few Smg6 -/- iPSC clones obtained were not fully reprogrammed and were not able to form teratomas or form chimeric mice when injected into mice or blastocysts, respectively.

Smg6 is an essential NMD factor and is also involved in telomere maintenance. In order to distinguish whether NMD or telomere maintenance was required for cell differentiation, the authors generated a set of Smg6 mutants that were selectively defective for NMD but still functioned in telomere maintenance and vice versa. The authors found that only the NMD-proficient Smg6 constructs were able to restore the differentiation capacity of ESCs when expressed ectopically, while the telomere-proficient but NMD inactive mutants failed to restore. This demonstrates that NMD rather than telomere maintenance is required for cell differentiation.

The authors then go on to show that c-Myc, one of the pluripotency factors is downregulated by NMD and that overexpression of c-Myc is sufficient to compromise ESC differentiation. Vice versa, knockdown of c-Myc in Smg6 -/- ESCs (in which c-Myc is elevated) alleviated the differentiation block. These experiments strongly suggest that c-Myc levels are normally downregulated by NMD and that this is a prerequisite for allowing differentiation. However, two crucial questions in this context remained unanswered: which features make c-Myc an NMD substrate and is the high c-Myc abundance in ESCs due to escape from NMD or due to an unrelated mechanism (e.g. transcriptional regulation) that boosts c-Myc levels despite of it being targeted by NMD. It might be revealing to check which isoform of c-Myc is expressed in ESCs and in EBs, since there is annotated in the NCBI database one mRNA isoform that starts from an upstream site and encodes in its 5' UTR a polypeptide that starts with a CUG and ends just uptstream of the main ORF. Thus an easily testable hypothesis is that in ESCs, the downstream transcription initiation site is used. This mRNA would not be expected to be an NMD substrate and the main ORF would be translated from this abundant mRNA producing the c-Myc protein that promotes pluripotency and inhibits differentiation. For differentiation to occur, transcription initiation would have to switch to the upstream site, producing the longer mRNA isoform in which the aforementioned upstream ORF would be the feature that triggers NMD and leads to low mRNA levels and hence low c-Myc protein. In NMD deficient cells, even the long mRNA isoform can reach high levels, produce high levels of c-Myc and thus prevent differentiation. I strongly encourage the authors to address this hypothesis, as it might bring them one significant step further into revealing the mechanism underlying the involvement of NMD in regulating embryonic differentiation.

Except from this and from few additional points listed below, this is a very exciting paper that will interest both the NMD community as well as developmental biologists since it reports a novel and important biological function of NMD. Overall, the experiments are well designed and controlled and hence the data and conclusions are compelling. Moreover, the paper is very well written and it was in fact a pleasure to read it.

Additional points:

Title: the authors may want to consider deleting "Telomerase cofactor" from the title and just say "Smg6/Est1A licenses embryonic ..."

Ensure that abbreviations are defined when they first appear in the text. This is currently not the case for example for ICM (p. 5) and EdU (p. 15).

p. 12 and Fig. S7C: The claimed reduction in c-Myc in Smg6 -/- ESCs after treatment of the cells with the TGF-beta inhibitor is not visible on this western blot.

Are the RNA samples used in Fig. 6A and the protein samples used in Fig. S8A from the same cells/experiments? How can it be that the average Upf1 mRNA level measured in three independent knockdown experiments is quite reproducible (6A), while the two protein samples show so drastically different Upf1 protein abundances (S8A; in one of the samples, there is no knockdown visible)?

Discussion: in 2011, Lawrence Gardner reported in JBC that overexpression of c-Myc inhibits NMD in B lymphocytes. I do not recall the details of this paper right now, but the authors might want to discuss these findings in the context of their own data. Is there any evidence for a feedback circuit?

Referee #3:

In their manuscript Li and colleagues identify the nonsense-mediated mRNA decay (NMD) pathway as essential to allow embryonic stem cell differentiation. The authors find that total deletion of Smg6 is embryonic lethal but embryonic stem (ES) cell lines with conditional deletion of Smg6 can be established. Smg6 knockout ES cells fail to undergo differentiation in a range of assays. The authors identify the NMD function of Smg6 as required to allow ES cell differentiation. Mechanistically, they then show that c-Myc is aberrantly expressed in Smg6 knockout cells and that inhibition of c-Myc allows differentiation in the absence of Smg6.

While the transcriptional regulation of self-renewal and exit of pluripotency is increasingly understood, only little is known about regulation of pluripotency through RNA processing enzyme. Here, the authors identify an essential role for the nonsense-mediated mRNA decay pathway in inducing ES cell differentiation. Importantly, a similar loss of ES cell differentiation has been described for the microRNA processing machinery including DGCR8 and Dicer (Wang et al. 2007 Nature Genetics and Kanellopoulou et al. 2005 Genes & Development). Therefore, I consider this manuscript as an important contribution to the field. One problem the authors face is the very severe effect on ES cells when Smg6 is deleted and the complete block of differentiation. The author's implication is that that self-renewal potential is unaffected but they do not provide sufficient evidence for this claim. In other words, are Smg6-knockput cells still ES cells or are they a transformed tumour-like counterpart? Mechanistically the authors show an important role for c-Myc, which would rather indicate a transformation of the cells since c-Myc is not anymore considered to be required or essential for self-renewal or reprogramming. In addition, the authors need to provide more quantifications and clarification of inconsistencies (see below).

Major comments

1. Page 6; line 1: The authors claim that deletion of Smg6 does not affect ES cell self-renewal and viability but do not provide evidence for this claim. At the very minimum a comparison of

proliferation, cell cycle and apoptosis should be provided. A chromosome spread would also provide evidence that the cells did not undergo major transformations.

2. Figure 1: The resistance of ESC to undergo differentiation in the absence of Smg6 is quite remarkable. The authors confirm that the protein levels of Oct4 remain high and do not detect markers of differentiation. While a similar resistance to differentiate has been observed when other RNA processing enzymes such as DGCR8 are deleted in ESC, the effect seems less dramatic and expression of differentiation markers was reduced or delayed rather than absent (Wang et al. 2007, Nature Genetics). The authors should provide quantifications for the observations in figure 1.

3. The only evidence provided that the ES cells maintain normal self-renewal is staining for Oct4. The authors should provide some more markers to confirm normal versus abnormal differentiation for at least one of the assays in a time course. For instance, what is the relative RNA abundance during differentiation in RA of differentiation (Ectoderm, endoderm, mesoderm, trophectoderm) versus undifferentiation (Nanog, Rex1, Sox2, Klf4, Tbx3, Tfcp2I1, Esrrb) markers?

4. In line with point 1: What happens to Smg6 knockout cells when they are stimulated to differentiate? Are they just inert or do they undergo apoptosis instead? Is number of ES cell colonies during the differentiation process higher when Smg6 is deleted? What is the percentage of undifferentiated and differentiated colonies in the absence and presence of LIF?

5. Re-programming is compromised in MEFs lacking Smg6. Does this not indicate that deletion of Smg6 does not simply promote a normal self-renewing and undifferentiated state? The authors should clarify this in the text.

6. Figure 4: The authors identify that the block of ES cell differentiation was due to Smg6's function in NMD. The author should provide evidence that all constructs are still equally expressed after 6 days of differentiation.

7. The rescue experiment could be seen as a prove that Smg6 knockout cells are not simply transformed if the authors provided some more evidence that the differentiation i.e. in RA in a time course shows marker expression similar to wild-type ES cells (see point 3).

8. To identify the potential mechanism of Smg6-blocked differentiation of ES cells, the authors perform RNA-seq experiments using wild-type and Smg6 knockout ES cells. The rationale for this experimental approach is unclear. Why do the authors analyse the undifferentiated ES cells when no phenotype or effect was observed? The authors further state that the global transcriptional landscape was unaffected by deletion of Smg6. However, recent data suggests that NMD affects the levels of 3-10% of all cellular mRNAs. Thus, does the RNA seq experiments indicate that NMD is not functional in wild-type ES cells? Have the authors analysed alternative splicing with respect to the inclusion or exclusion of premature termination codon (PTC)-containing cassettes?

9. Alternatively, NMD may function more specifically in ES cells and c-Myc RNA which is usually degraded by NMD can now be stabilized. It is puzzling however, that the very high up-regulation of c-Myc on RNA and protein levels does (i) not cause more transcriptional changes than described in Figure 5a and (ii) not affect proliferation, apoptosis or morphological differences (see page 6; line 1). The authors should strengthen their claims by showing some validated c-Myc target genes and cell cycle analysis.

10. The authors should use the RNA seq data to support their claims. Yet, the data provided in Table S1 do not show any changes in Oct-4, Sox2 or Rex1 but down-regulation of Nanog. Transcription factors that seem highly up-regulated are Sox17 and Gata6, both of which are considered to be a marker for primitive endoderm.

1st Revision - authors' response 13 January 2015

Response to Reviewers – Overview of Revisions on Figures

Figure	Revision	Text	Content	Reviewer(s)
Fig. 1	New Panel B	Page 5, parag. 2, bottom line 2	$Smg6^{\Delta/\Delta}$ ESC morphology	#3, point 4
	New Panel C	Page 5, parag. 2, bottom line 2	Proliferation of $Smg6^{\Delta/\Delta}$ ESCs	#1, point 4 #3, point 1, 3
	New Panel D	Page 6, parag. 1, line 3	Apoptosis of $Smg6^{\Delta/\Delta}$ ESCs	#1, point 4 #3, point 1
	Panel I	Page 7, parag. 2, line 4	Added quantification	#3, point 2
Fig. 3	Panel B	Page 9, parag. 3, bottom line 2	Added more NMD targets	#1, point 2
	New Panel C, D	Page 10, parag. 3, bottom line 1	Analysis of PTC+ targets	#3, points 8
Fig. 5	New Panel E, F, G	Page 13, parag. 3, line 3	Analysis of c-Myc isoforms	#2, main point
Fig. S2	New Panel F	Page 6, parag. 1, line 1	Cell cycle profile	#1, point 4 #3, point 1, 3
	New Panel G	Page 9, parag 2, line 4	Karyotype of $Smg6^{\Delta/\Delta}$ ESCs	#3, point 1
Fig. S3	New Panel C	Page 6, parag 2, line 6	Added quantification	#3, point 2
	New Panel D	Page 6, parag 2, line 12	EBs size increase	#3, point 4
	New Panel E	Page 6, parag 2, line 14	EdU+ cells in EBs	#3, point 4
	New Panel F	Page 6, parag 2, line 16	Apoptosis of $Smg6^{\Delta/\Delta}$ EBs	#1, point 5 #3, point 4
Fig. S5	New Panel C	Page 10, parag 3, line 4	Western blot analysis of expression of Smg6 constructs	#1, point 3 #3, point 6
	New Panel D, E	Page 10, parag. 3, bottom line 1	Analysis of PTC+ targets	#3, points 8
	New Panel G	Page 11, parag. 2, bottom line 5	Analysis of stemness and differentiated genes in ESCs and EBs	#3, points 3
Fig. S6	New Panel B	Page 13, parag. 1, line 3	Expression of c-Myc targets	#3, points 9 #1, point 5
	New Panel D	Page 14, parag 2, line 4	c-Myc overexpression on NMD targets	#1, minor point 6
	Panel E	Page 14, parag 2, line 12	Replacement by a new panel	#2, additional point 3
Fig. S7	New Panel B	Page 15, parag. 2, bottom line 3	c-Myc expression after NMD factor knockdown	#1, point 1

Reply to Referee #1:

Li et al. is an intriguing study providing strong evidence that a post-transcriptional pathway promotes both the differentiation and generation of pluripotent stem cells. This discovery fills an important niche, as the vast majority of work in the field of pluripotent cell differentiation/reprogramming has, instead, been focused on transcriptional pathways. The authors show several lines of evidence that the RNA degradation pathway, NMD, is responsible for these effects on pluripotent cells (e.g., by knocking down different NMD factors) and they elegantly rule out another possibility - that the telomerase-promoting activity of NMD factors is responsible. Importantly, they also identify a particular transcript - c-myc mRNA - that NMD downregulates to promote ES differentiation. Together, this study represents a major step forward towards understanding the underlying molecular mechanisms responsible for going to-and-from the pluripotent state.

While I have a lot of enthusiasm for this manuscript, there are some issues that must be dealt with, both major and minor.

We appreciate this reviewer's positive view on our study and thank him/her for the constructive comments. We agree with the comments and followed the suggestions for improvement. Detailed responses to the comments are provided under each point in blue. Please also note that we have underlined the modifications in the revised manuscript.

Major Issues:
(1) c-myc is a NMD target. Since the authors are claiming that they have identified a NMD circuit involving c-myc mRNA, it is absolutely essential that they demonstrate that this transcript is indeed a NMD target. At the very least, the authors must (i) test whether c-myc mRNA is not only upregulated by SMG6 knockdown, but also in response to KD of 1 or more other NMD factors (since SMG6 has activities in addition to NMD), (ii) test whether c-myc mRNA is stabilized by SMG6 KD (since the hallmark of NMD is that it destabilizes its targets), and (iii) identify at least one feature in c-myc RNA that could be responsible for it being targeted by NMD pathway; e.g., an intron in the 3′ UTR, a long 3′ UTR, and an uORF are all known NMD-inducing features. In addition, it would be preferable (but not required) if the authors empirically tested whether this feature triggers NMD. It would also strengthen this manuscript if the authors tested whether an NMD-insensitive form of c-myc mRNA prevents mES cell differentiation. Of note, if the authors find that c-myc mRNA is NOT a direct NMD target, that is ok, but then they should investigate (at least to some degree) the underlying mechanism by which NMD indirectly regulates c-myc (e.g., is it transcriptionally or post-transcriptionally regulated by NMD?).

This is an important point. Following his/her suggestion, we have performed all experiments to address the comments. (i) We have analysed the c-Myc mRNA level after the knockdown of other NMD factors and found that the c-Myc mRNA level is indeed up-regulated in shUpf1- and shUpf2-transfected ESCs (Rev Suppl Fig S7B). (ii) and (iii) We performed a c-Myc 3'UTR reporter luciferase assay (Kumar et al. Nat Genet, 2007) and found that c-Myc 3'UTR is stabilised in Smg6 mutant cells (Rev Fig 5G). In addition, the data-mining of our RNA-seq data revealed that all the transcripts of c-Myc isoforms containing 3'UTR were increased in mutant ESCs (Rev Fig 5E, 5F, see also our response to reviewer 2). Finally, as shown in the original manuscript, we stably overexpressed the GFP-c-Myc (lacking 3'UTR and 5'UTR, thus insensitive to NMD) in ESCs. These ESCs are defective in differentiation, phenotypically mimicking Smg6 null ESCs, thus indicating that NMD insensitive form of c-Myc can prevent ESC differentiation.

(2) Endogenous NMD targets. The NMD target transcripts the authors have chosen to measure NMD magnitude in response to loss of SMG6 are either also induced by stress (e.g., ATF4), which complicates interpretation of the results, or are non-coding RNAs (e.g., GAS5), which may be degraded by NMD in an unconventional way since they lack a long ORF. Therefore, the authors should also include well-characterized protein-coding NMD substrates mRNAs that are not linked with stress.

This point is well taken. We now analysed by qRT-PCR the gene transcripts that are stress-independent, such as Smg1, Smg5, Upf1 and Upf2 (Huang et al. Mol Cell, 2011), Auf1 and Hnrnp1

(http://www.ensembl.org/Mus_musculus/Info/Index) in Smg6 deficient ESCs. All of these gene transcripts were up-regulated (Rev Fig 3B).

(3) Exogenous SMG6. The levels of exogenous SMG6 (in its various form) in the rescue experiments must be shown. It is important to know their level relative to endogenous SMG6 to fully interpret the experiments.

We used the GFP-fluorescence intensity to quantify the expression levels of exogenous Smg6 constructs in the original manuscript (Orig Suppl Fig S6B). As suggested (also by Reviewer 3), we further analysed the expression of the exogenous proteins in ESCs and EBs by Western blotting and found similar expressions among the constructs (Rev Suppl Fig S5C). Interestingly, we noticed that the Smg6 protein seems to be increased in EBs compared to that in ESCs, which suggests a role for Smg6 in the differentiation. However, since the Smg6 antibody is raised against the PIN domain, we could not detect the expression of the ΔPIN-Smg6 construct. Nevertheless, given the FAC analysis of the GFP fluorescence intensity and a similar biological phenotype between Δ14-3-3 and ΔPIN-Smg6, we can rule out any artefacts that influenced the biological effect in the rescue experiments.

(4) Effect of loss of SMG6 in ESCs. On the bottom of pg. 5, it is indicated that loss of SMG6 has no effect on proliferation or morphology, but no quantification of passage numbers or proliferation rate is provided. Related to this, the authors claim that there are little or no telomeres in SMG6-mutant cells; this seems incompatible with survival, and thus should be addressed.

This is an important point. To address this concern, we have measured the proliferation of Smg6-null ESCs for 12 passages and found no difference compared to the controls (Rev Fig 1C). Further, BrdU pulse-labelling revealed no difference between the cell cycle profiles of Smg6 mutant ESCs and controls (Rev Suppl Fig S2F). Finally, we did no detect any differences in cell death by Annexin-V FACS analysis (Rev Fig 1D).

Although the telomere loss compromises cell proliferation in primary somatic cells, it apparently does not compromise ESC viability. For example, Tert-/- ESCs can be maintained despite a progressive loss of telomere length and genomic instability (Wang et al. Proc Natl Acad Sci, 2005). It should be noted that most, if not all, of our knowledge on telomere biology comes from somatic cells and adult stem cells, where limited proliferations are expected (Blasco, Nat Chem Biol, 2007). Proliferation and cell death are triggered by telomere shortening mediated DNA damage response, involving the ATM and p53 pathways (Karlseder et al. Science, 1999; Herbig et al. Mol Cell, 2004; Sperka et al. Nat Cell Biol, 2012). ESCs may harbour a different mechanism to handle telomere integrity-mediated cell proliferation and the death programme. For example, ESCs are characterised by a short G1 and lack a strong cell cycle checkpoint (Reviewed by Orford & Scadden, Nat Rev Genet, 2008; Abdelalim, Stem Cell Rev, 2013). We have included the discussion of this point in the revised manuscript (page 16, parag. 1).

(5) NMD, c-myc, and proliferation. There is a vast literature showing that c-myc promotes proliferation and also some literature showing the same for NMD. Thus, it is surprising that the authors do not find that loss of the NMD factor, SMG6, has much of any effect on proliferation. The authors should address this. They should also address the fact that NMD opposes the expression of c-myc and thus one might expect that NMD and c-myc would serve opposite roles. In the case of embryoid bodies, they refer to Smg6-mutant cells proliferating more than control cells, but do not show the data (pg. 15). They should show the data and discuss it in light of the previously known roles of NMD and c-myc in proliferation. Likewise, they should also experimentally address (using FACS and/or EdU labeling) or at least discuss the role of NMD/c-myc in undifferentiated ES cells and MEFs.

This point is also touched by reviewer 3 (point 9). c-Myc overexpression has been well documented in somatic studies of cell proliferation by transcriptionally regulating cell proliferation genes (see reviews by Cole, Ann Rev Genet 1986; Dang et al. Mol Cell Biol, 1999; Pelengaris et al. Nat Rev Cancer, 2002; Eilers & Eisenman, Genes Dev, 2008). NMD factors have also been implicated in regulating the proliferation of somatic cells or tissue progenitors (McIlwain et al. Proc Natl Acad Sci, 2010; Hwang & Maquat, Curr Opin Genet Dev, 2011; Lou et al. Cell Reports, 2014). These literatures are based on somatic cells or transient knockdown experiments. Surprisingly, we found that c-Myc overexpression and Smg6 knockout do not compromise ESCs proliferation. To the best

of our knowledge, these are the first observations on c-Myc overexpression or NMD knockdown in ESC proliferation. Apparently, ESC proliferation is less sensitive to a change of c-Myc or NMD activity. It is an interesting observation; however, the underlying mechanism accounting for the discrepancy is unknown but beyond the scope of the current study. We have added a discussion on c-Myc, NMD in cell proliferation in the revised manuscript (page 19, parag. 2).

Regarding the increased cellularity in Smg6 mutant EBs mentioned in our original manuscript, this was based on the density (in histology) and the higher number of EdU+ cells in mutant EBs ("data not shown" in the original manuscript). Now, we added these data (Rev Suppl Fig S3E). The high cellularity and the high rate of EdU labelling in the mutant EBs reflects their high content of undifferentiated cells, in contrast to wild-type EBs, which contain mostly differentiated cells. We have now clarified this point in the revised manuscript (page 17, parag. 2).

(6) Fig. 1F. It would be preferable if lineage-specific markers were used to definitively assess the germ layer defects.

The original Fig 1F showed a GFP-positive cell distribution (derived from ESCs injected) in the tissues of chimeric foetuses. Because the origin of these structures is well documented and these are well-established assays to examine the ESC derivatives *in vivo*, we feel it is not necessary to verify the origin of these tissues (as also recommended by the Editor). Nevertheless, in our *in vitro* assay, we stained the EBs with antibodies against different germ layer markers, such as Nestin (ectoderm), β-Catenin (endoderm), α-SMA (mesoderm). The data was documented in the original Suppl Fig. S3C. However, it seems to be hidden (and it was also missed by reviewer 3 (point 3)). We now move these data to the main figure (Rev Fig 1G).

(7) Fig. 2A. The Smg6-null cells appear to be considerably smaller than the control cells. This should be noted, along with speculation as to why this might be so.

We assume that the "smaller" size in this image that the reviewer referred to is due the iPSC colony size (original page 8). We speculate that this phenomenon may be attributable to an inefficient iPSC production from Smg6 mutants, which contain less proliferating iPSCs and thus smaller colonies. Now, we discussed this point in the revised manuscript (page 8, parag. 3).

(8) c-myc targets. It would elevate this manuscript if the authors identified the complete set of candidate genes that NMD regulates through c-myc. This can easily be done by mining known c-myc target genes (defined from published databases) as to whether they correspond to transcripts dysregulated in the authors' Smg6-mutant RNA-seq datasets.

We are very grateful for this suggestion. We have correlated the c-Myc targets (from Chen et al. Cell, 2008) with our Smg6 DEGs. We identified that 95 out of 2449 Smg6 DEGs are regulated by c-Myc and now documented them in Rev Suppl Table S5. Although lacking a correlation between Smg6-DEGs and the c-Myc target ($P = 0.99$, Hypergeometric Test), these data may not be surprising, because NMD should have a general role in regulating RNA pools. For example, NMD plays a role in the post-transcriptional regulation of gene transcripts, while c-Myc transcriptionally regulates the gene expression by directly binding to promoters (Dang et al. Mol Cell Biol, 1999; Lin et al. Cell, 2012; Nie et al. Cell, 2012). We have included these data in the revised manuscript (page 13, parag. 1; Rev Suppl Table S5).

Minor Issues:
(1) Fig. 5B. It is critical that all functional categories that are statistically over-represented ($P<0.05$) are shown in this figure. The precise P values should also be indicated. Finally, the authors should provide not only the numerator (# proteins corresponding to mRNAs dysregulated) but also the denominator (the number of proteins in a given category).

In our GO analysis, we first used DAVID to obtain significant GO terms from our DEG list. GO terms with $P < 0.05$; FDR < 0.05 are used to construct the enrichment map (Orig Fig 5B). The enrichment map was constructed using Cytoscape and installed with the Enrichment Map plug-in. Each enriched GO pathway is represented by a red node. In this figure, we did not intend to show specific GO terms. However, the GO pathways with similar functions are sorted into one cluster, marked with a specific description in the label. Meanwhile, the gene number in each cluster here is

calculated by all genes with a similar GO function (within the same circle). Now in the revised manuscript, we included a new supplemental table (Rev Suppl Table S3) to document the number of each significant GO term in our DEGs list and in the total gene background. We hope this will help readers to extract their information more precisely.

(2) There are several over-statements in this manuscript, which need to be toned down. For example, in the Abstract, it says that lethality as a result of NMD factor loss is caused by defects in homeostasis. This is not necessarily true, so the word "indicates" is too strong.

We have checked and toned down the overstatements.

(3) This manuscript needs major copy-editing. A few examples include: (i) Abstract: the first and second sentences have no transition; (ii) Abstract, last line, should be changed to something like: "...both the differentiation of stem cells and the reprogramming of differentiated cells into stem cells;" (iii) Pg. 4, first sentence of first full paragraph: "involved," not "involves;" and (iv) Pg. 13, near top: insert "other" between "knock down" and "NMD factors." This is only a partial list; the entire manuscript needs to be carefully scrutinized.

We appreciate this reviewer's specific comments and have tried to improve these sentences. See below.

(4) Correct word usage. In addition to improvements in grammar and style, more appropriate word usage should be implemented. For example, on pg. 4, "...may exaggerate the complexity...in vivo" should be replaced with something like "...complicates the interpretation of phenotypic effects that result from their loss." On pg. 9, first sentence of the first full paragraph, the word "genes" should be replaced with "RNAs" or "transcripts," as NMD acts on RNAs, not genes (also see the same error in the next paragraph). As another example, on pg. 11, RNA-seq analysis does not measure "transcriptional levels," it measures "RNA levels." Just below that, it states that "the global transcriptional profile did not change...," when in fact the RNA prolife (note, not the "transcriptional profile") DID change (>2000 genes changed in level!); phrasing needs to be altered to reflect a general similarity in RNA profile but statistically significant changes in ~10% of the transcriptome. The entire manuscript should be carefully screened for these kinds of errors.

Regarding points (3) and (4), we have asked a native speaker to go through the manuscript again and we carefully double-checked and corrected those errors or inaccurate wording.

(5) It is strongly suggested to mention that the Smg6-mutant ESCs and MEFs have a deficiency in NMD when these mutant cells are first discussed. Refer to data that will be presented later.

As suggested, we stated in "Discussion" that "Smg6 knockout ESCs and MEFs are viable but NMD deficient" (page 16, parag. 2).

(6) The Discussion requires a more in-depth analysis of previously published studies showing a connection between NMD and proliferation/cancer; e.g., Wang et al. MCB '11 previously showed that c-myc inhibits NMD (which coupled with the data in the present paper, suggests a mutually enforcing feedback loop) and Liu et al. Nature Medicine '14 showed that inactivating mutations in the NMD gene, UPF1, are likely to cause a specific type of cancer.

Wang et al. (J Biol Chem 2011) reported that an overexpression of c-Myc in B cells inhibits NMD and thus stabilises the NMD targets. We show that the high c-Myc level could be a consequence of an NMD deficiency. However, we did not detect an obvious inhibition of NMD targets when c-Myc was overexpressed. There is no evidence of a c-Myc-NMD feedback loop in ESCs. As suggested, we have added this in our discussion (page 19, parag. 2). Also we have added many points for discussion in responding to the reviewers' comments and suggestions.

(7) The authors should comment on whether or not it is known if KO or KD of telomerase factors besides NMD factors are required for ES cell differentiation.

There are only limited reports on the effect of telomere knockout on ESC differentiation. Pucci et al. (Cell Stem Cell 2013) used very late passages (p67 to p74) of mTerc-/- ESC to conduct a

differentiation assay and found that only the late passages of ESCs showed differentiation defects. As suggested, we include this point in the discussion (page 17, parag. 2).

Reply to Referee #2:

We appreciate this reviewer's positive view of our data. We have addressed all comments in the revised manuscript and also provide a detailed explanation below (in blue). Please note that we have underlined the modifications in the revised manuscript.

"Li and colleagues have investigated here the effect of Smg6 ablation on embryonic cells' capacity to differentiate...

"Smg6 is an essential NMD factor and is also involved in telomere maintenance. ...

"The authors then go on to show that c-Myc, one of the pluripotency factors is downregulated by NMD and that overexpression of c-Myc is sufficient to compromise ESC differentiation…

"Except from this and from few additional points listed below, this is a very exciting paper that will interest both the NMD community as well as developmental biologists since it reports a novel and important biological function of NMD. Overall, the experiments are well designed and controlled and hence the data and conclusions are compelling. Moreover, the paper is very well written and it was in fact a pleasure to read it. "

We are grateful for this reviewer's appreciation of our work. The c-Myc mRNA regulation is an important and yet a complicated mechanism in stem cell biology. We thank this reviewer for his offer of a possible mechanistic explanation by which c-Myc transcripts are regulated in ESC maintenance and differentiation. These are important comments.

We followed his/her suggestion and investigated the isoforms of c-Myc in ESCs and in differentiation.
(1) We re-analysed the RNA-seq data and found one long isoform (mRNA accession number ENSMUST00000167731, encoding a 453 aa protein) and two short isoforms (mRNA accession number ENSMUST00000161976, and ENSMUST00000160009, both encoding a 439 aa protein) in control ESCs. These are all over-represented in Smg6 mutant ESCs (Rev Fig 5E, 5F).
(2) A Western blot analysis revealed that in controls these isoforms disappeared upon EB differentiation. However, they maintained still high in Smg6 mutant ESCs and EBs (Rev Fig 4F, 5F). This strongly suggests that c-Myc long and short isoforms are specific targets of NMD in ESCs.
(3) We found that 3'UTR of the c-Myc is responsible for the Smg6-mediated c-Myc mRNA stability (Rev Fig. 5E, 5G).

These new data prove the hypothesis of this reviewer and suggest that Smg6 regulates the stability of c-Myc in ESCs and EBs, a disturbance of which compromises the differentiation process. Further support comes from our original data showing that an ectopic expression of NMD insensitive c-Myc (GFP-c-Myc) prevents the differentiation of ESCs (Rev Fig 5H). All these new data are included in the revised manuscript.

Additional points:
Title: the authors may want to consider deleting "Telomerase cofactor" from the title and just say "Smg6/Est1A licenses embryonic ..."

Since the original name of Smg6, Est1, and the function of Smg6/Est1 were well described in telomere mutant yeast, our study indeed demonstrates the telomere independent but NMD dependent function of Smg6/Est1 in ESC differentiation. It would be attractive for a wider readership if we kept the "Telomerase cofactor" in the title.

Ensure that abbreviations are defined when they first appear in the text. This is currently not the case for example for ICM (p. 5) and EdU (p. 15).

Done.

p. 12 and Fig. S7C: The claimed reduction in c-Myc in Smg6 -/- ESCs after treatment of the cells with the TGF-beta inhibitor is not visible on this western blot.

We have replaced this figure (Rev Suppl Fig S6E) by a lower exposure and now the reduced c-Myc level is visible.

Are the RNA samples used in Fig. 6A and the protein samples used in Fig. S8A from the same cells/experiments? How can it be that the average Upf1 mRNA level measured in three independent knockdown experiments is quite reproducible (6A), while the two protein samples show so drastically different Upf1 protein abundances (S8A; in one of the samples, there is no knockdown visible)?

The original Fig 6A and Fig S8A (now Rev Suppl Fig S7A) were indeed from the same cells. qRT-PCR was performed in three clones of shUPF1 knockdowns and two of them were used for a Western blot analysis. Although Western blotting from these two clones showed a variation in knockdown efficiency, a qRT-PCR analysis from the three clones indeed showed defects in NMD target decay. We only found the variation in the UPF1 knockdown at the protein level, which may reflect the survival pressure (Azzalin & Linger, Curr Biol, 2006) that selects strongly against knockdown efficiency.

Discussion: in 2011, Lawrence Gardner reported in JBC that overexpression of c-Myc inhibits NMD in B lymphocytes. I do not recall the details of this paper right now, but the authors might want to discuss these findings in the context of their own data. Is there any evidence for a feedback circuit?

A similar comment was also raised by reviewer 1 (minor point 6). The Gardner Lab reported that an overexpression of c-Myc in B cells inhibits the NMD and thus stabilises the NMD targets (Wang et al. J Biol Chem 2011). However, this inhibition was not evident in the ESCs (Rev Suppl Fig S6D). We have discussed this (page 19, parag 2).

Reply to Referee #3:

"*In their manuscript Li and colleagues identify the nonsense-mediated mRNA decay (NMD) pathway as essential to allow embryonic stem cell differentiation. ...*

"While the transcriptional regulation of self-renewal and exit of pluripotency is increasingly understood, only little is known about regulation of pluripotency through RNA processing enzyme. ...

"Mechanistically the authors show an important role for c-Myc, which would rather indicate a transformation of the cells since c-Myc is not anymore considered to be required or essential for self-renewal or reprogramming. In addition, the authors need to provide more quantifications and clarification of inconsistencies (see below). "

This reviewer appreciates the importance of the work, but raised questions and suggestions to improve it. We have performed new experiments and given explanations (see below in blue) to address all points and we provided new data in the revised manuscript. Please note that all modifications are underlined in the revised manuscript.

Major comments
1. Page 6; line 1: The authors claim that deletion of Smg6 does not affect ES cell self-renewal and viability but do not provide evidence for this claim. At the very minimum a comparison of proliferation, cell cycle and apoptosis should be provided. A chromosome spread would also provide evidence that the cells did not undergo major transformations.

The point is well taken (which is also raised by reviewer 1). To address their concerns, we examined the proliferation of mutant ESCs (for 12 passages), and analysed the cell cycle profile (by FACS analysis after BrdU-PI straining) and apoptosis (by Annexin-V staining). We did not detect any differences in these parameters between control and $Smg6^{\Delta/\Delta}$ ESCs (Rev Fig 1C, 1D, Suppl Fig

S2F).

To address the concern that the Smg6-null ESCs are transformed and hence cannot differentiate, our strongest evidence is the rescue experiment, where rescued ESCs can form differentiated cells/tissues in culture and in chimera assays (Rev Fig 4), which is also appreciated by this reviewer (see point 7). In addition, we performed a karyotype analysis and found no obvious difference between control and Smg6$^{\Delta/\Delta}$ ESCs (Rev Suppl Fig S2G). This point is important and we now also discussed and clarified it in the revised manuscript (page 17, parag. 2).

2. Figure 1: The resistance of ESC to undergo differentiation in the absence of Smg6 is quite remarkable. The authors confirm that the protein levels of Oct4 remain high and do not detect markers of differentiation. While a similar resistance to differentiate has been observed when other RNA processing enzymes such as DGCR8 are deleted in ESC, the effect seems less dramatic and expression of differentiation markers was reduced or delayed rather than absent (Wang et al. 2007, Nature Genetics). The authors should provide quantifications for the observations in figure 1.

A quantification of the original Fig 1B is shown in the original Fig 4C. The original Fig 1F was summarised in Table 1. Now, the quantification of the original Fig 1D and 1E is included in the revised manuscript (page 7, parag. 2; Rev Fig 1I, Suppl Fig S3C).

3. The only evidence provided that the ES cells maintain normal self-renewal is staining for Oct4. The authors should provide some more markers to confirm normal versus abnormal differentiation for at least one of the assays in a time course. For instance, what is the relative RNA abundance during differentiation in RA of differentiation (Ectoderm, endoderm, mesoderm, trophectoderm) versus undifferentiation (Nanog, Rex1, Sox2, Klf4, Tbx3, Tfcp2I1, Esrrb) markers?

In the original manuscript, we analysed the stemness markers Oct4, Nanog, c-Myc (Orig Fig 1C and 4E) and the differentiation markers Nestin, β-catenin and α-SMA (Orig Suppl Fig S3C and Orig Fig 4F) in EB formation assays. Because the latter is hidden and is also missed by reviewer 1 (point 6), we moved these data to the main figure (Rev Fig 1G). We now provide more evidence on the self-renewal of Smg6-deficient ESCs by analysing the proliferation, the cell cycle profile and cell death (Rev Fig 1C, 1D, Rev Suppl Fig S2F, S3D, S3E, S3F).

To follow this reviewer's suggestion, we analysed the expression of the stemness markers (Esrrb, Oct4, Nanog, Rex1) and of the lineage markers (Mixl1, Pax6, Foxa2) in ESCs, and EBs at day 5 and day 8. We found that the expression levels of the stem cell factors are higher in Smg6-deficient EBs at day 5, which can be efficiently repressed by the expression of NMD-proficient Smg6 vectors, suggesting that they are under the control of NMD. We also noticed a high expression of differentiation gene transcripts in Smg6-NMD-deficient EBs. The expression of differentiation genes (also shown in Rev Suppl Fig S5G) is not sufficient to drive ESC differentiation. These findings nevertheless are consistent with the general notion that a failure to repress the stemness gene expression disturbs the differentiation program (see Gu et al. Mol Cell Biol, 2005; Ivanova et al. Nature, 2006; Gabut et al. Cell, 2011; Han et al. Nature, 2013; or see review by Keller, Genes Dev, 2005). We included these data in the revised manuscript (Rev Suppl Fig S5G) and also in the discussion (page 18, parag. 1).

4. In line with point 1: What happens to Smg6 knockout cells when they are stimulated to differentiate? Are they just inert or do they undergo apoptosis instead? Is number of ES cell colonies during the differentiation process higher when Smg6 is deleted? What is the percentage of undifferentiated and differentiated colonies in the absence and presence of LIF?

To identify the cellular fate of Smg6$^{\Delta/\Delta}$ ESCs under differentiation conditions, we investigated the cell death in EBs on day 5 by staining with cleaved Caspase-3 and TUNEL and found a similar cell death in Smg6 knockout and control EBs (Rev Suppl Fig S3F). Smg6$^{\Delta/\Delta}$ ESCs in EB differentiation assays were not inert, but showed a higher frequency of EdU incorporation (Rev Suppl Fig S3E). Indeed, we noticed a gradual increase of Smg6$^{\Delta/\Delta}$ EB size during differentiation yet less than their wild-type counterparts (Rev Supp Fig S3D). When cultured in undifferentiation conditions, control and Smg6 null ESCs all exhibited ESC morphology (Rev Fig 1B), whereas in feeder-free and LIF-free conditions (to induce differentiation), Smg6 null cultures still maintained a higher number of ESC like colonies (page 6, parag. 2; Rev Suppl Fig S3C).

5. Re-programming is compromised in MEFs lacking Smg6. Does this not indicate that deletion of Smg6 does not simply promote a normal self-renewing and undifferentiated state? The authors should clarify this in the text.

Agreed. We claim that "*Smg6-NMD per se is not required for the steady-state and self-renewal of stem or somatic cells, but is a licensing factor for cell identity switching during differentiation or reprograming processes.*" (page 18, parag. 1).

6. Figure 4: The authors identify that the block of ES cell differentiation was due to Smg6's function in NMD. The author should provide evidence that all constructs are still equally expressed after 6 days of differentiation.

In the original Fig S6B, we used a FACs analysis to quantify the expression of stably transfected GFP-tagged vectors, where a very similar expression level was found among the transfectants. Now, we have added Western blotting data on the ectopically expressed vectors in ESCs and we differentiated EBs at day 6 (Rev Suppl Fig S5C). These vectors seem to express stably during differentiation.

7. The rescue experiment could be seen as a prove that Smg6 knockout cells are not simply transformed if the authors provided some more evidence that the differentiation i.e. in RA in a time course shows marker expression similar to wild-type ES cells (see point 3).

Indeed, the rescued ESCs can restore their capacity to differentiate *in vitro* (spontaneous differentiation and EB assays) and more importantly *in vivo* to form various tissues in chimera assays (Rev Fig 4). These are strong evidence that Smg6 null ESCs are not transformed. In addition, we found in these rescued ESCs restored the normal expression pattern of differentiation markers in EBs (see Rev Fig 4G). Please also see our response to point 1 and 3.

8. To identify the potential mechanism of Smg6-blocked differentiation of ES cells, the authors perform RNA-seq experiments using wild-type and Smg6 knockout ES cells. The rationale for this experimental approach is unclear. Why do the authors analyse the undifferentiated ES cells when no phenotype or effect was observed? The authors further state that the global transcriptional landscape was unaffected by deletion of Smg6. However, recent data suggests that NMD affects the levels of 3-10% of all cellular mRNAs. Thus, does the RNA seq experiments indicate that NMD is not functional in wild-type ES cells? Have the authors analysed alternative splicing with respect to the inclusion or exclusion of premature termination codon (PTC)-containing cassettes?

The RNA-seq analysis on ESCs was originally designed to address three questions: (1) Why do Smg6 null ESCs not die, which is unexpectedly given that NMD factors have been linked to cellular viability? (2) To find possible NMD targets that are altered, which may disturb the pluripotency programme; and (3) Whether telomere maintenance genes are affected, which may contribute to the differentiation programme. Our original sentence "global transcriptional landscape was unaffected by the deletion of Smg6" may be misleading. What we meant to say is that the Smg6 deletion did not cause chaotic changes in the whole transcriptome. We have now rephrased the sentence. Indeed, our RNA-seq data indicates that ~9.5% of the gene transcripts are altered after Smg6 deletion, which is consistent with the prediction of 3-10% changes as mentioned by this reviewer and by Schweingruber et al. (Biochim Biophys Acta, 2013). We now included this information in the revised manuscript (page 12, parag. 2).

In the original Fig 3C, we showed by a GFP-based PTC reporter assay an abnormally higher expression of the PTC-containing vector in Smg6-deficient ESCs. To address the suggestion by this reviewer (which is also raised by reviewer 1, point 2), we further analysed the alternative splicing coupled NMD (AS-NMD) (Weischenfeldt et al. Genome Biol, 2012). As shown in Rev Fig 3C, 3D, the Smg6 deletion significantly increased the NMD target transcripts with features of exon-inclusion and exon-exclusion. To further investigate whether these increases are dependent on Smg6-NMD, we analysed these AS-NMD events in rescued clones and found that an expression of NMD proficient constructs (i.e. ΔN-Smg6 and FL-Smg6) can efficiently repress the expression of these AS-NMD isoforms (Rev Suppl Fig S5D, S5E). Thus, Smg6 is active for the AS-NMD in ESCs. We have added these new data in the revised manuscript (page 9, parag. 3; page10, parag. 3).

9. Alternatively, NMD may function more specifically in ES cells and c-Myc RNA which is usually degraded by NMD can now be stabilized. It is puzzling however, that the very high up-regulation of c-Myc on RNA and protein levels does (i) not cause more transcriptional changes than described in Figure 5a and (ii) not affect proliferation, apoptosis or morphological differences (see page 6; line 1). The authors should strengthen their claims by showing some validated c-Myc target genes and cell cycle analysis.

This point is also related to the response to point 1. Based on somatic cell studies, a c-Myc overexpression often affects cell proliferation and cell death (Cole, Ann Rev Genet 1986; Dang et al. Mol Biol Cell, 1999; Adhikary & Eilers, Nat Rev Mol Cell Biol, 2005; Li et al. Proc Natl Acad Sci, 2003; Srinivasan et al. Cell Reports, 2013). In the revised manuscript we analysed the proliferation, the cell cycle profile and apoptosis. It may be surprising that a c-Myc overexpression does not affect the proliferation, apoptosis and transformation of Smg6-deficient ESCs. However, to the best of our knowledge, there is no report on a c-Myc overexpression inducing ESC transformation. The function of c-Myc in ESCs and tissue stem cell compartments is often debatable.

After an analysis of the c-Myc targets (Chen et al. Cell, 2008) (Reviewer 1 also suggests to mine the RNA-seq dataset) in our Smg6 DEGs dataset, we identified that 95 out of 2449 Smg6 DEGs are regulated by c-Myc (Rev Suppl Table S5). Many cell cycle and apoptosis genes are well-known targets of c-Myc (Dang et al. Mol Cell Biol, 1999; also suggested by this Reviewer). We found, however, that these genes are not significantly changed in Smg6 null ESCs (Rev Suppl Fig S6B). Consistent with this finding, our new cell cycle analysis revealed no difference of cell cycle profile in mutant ESCs compared to controls (Rev Suppl Fig S2F). The functional difference of c-Myc in the proliferation genes of ESCs and somatic cells is not known, but it may be due to cell type specificity or a threshold of c-Myc expression. We have discussed this in the revised manuscript (page 19, parag. 2, bottom).

10. The authors should use the RNA seq data to support their claims. Yet, the data provided in Table S1 do not show any changes in Oct-4, Sox2 or Rex1 but down-regulation of Nanog. Transcription factors that seem highly up-regulated are Sox17 and Gata6, both of which are considered to be a marker for primitive endoderm.

We found a down-regulation of *Nanog* and an up-regulation of *Sox17* and *Gata6* in the Smg6-mutant RNA-seq dataset. Although Smg6-deficient ESCs express high levels of Sox17 and Gata6, they failed to differentiate into the endoderm. Likewise, the expression of differentiation genes (also shown in Rev Suppl Fig S5G) is not sufficient to drive differentiation. This differentiation failure could be due to a high level of c-Myc (see Rev Fig 4, Fig 5), which can repress the primitive endoderm differentiation as reported by Smith et al (Cell Stem Cell, 2010) or due to a repression of other stemness genes (Rev Suppl Fig S5G). We have also discussed this in our response to point 3.

2nd Editorial Decision 10 February 2015

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by two of the original referees (comments included below) and as you will see they both find that all criticisms have been addressed and support publication, pending minor final revision as outlined below.

As you will see from the reports, ref #1 asks you to comment on the basis for referring to the 3'UTR of the myc mRNA as 'driving mRNA instability' - and to provide further data on the destabilizing effect relative to a control 3'UTR. If you already have such data at hand I would encourage you to include it in the manuscript, but from our side - and given the otherwise positive input from both referees - additional experimental insight on this point will not be absolutely required (however, you should still comment on the concern raised here).

Regarding the abstract, I have included my suggestion for an edited version below, could you let me know if you would agree to it?

Nonsense-mediated mRNA decay (NMD) is a post-transcriptional mechanism that targets defective transcripts and regulates the cellular RNA reservoir. Genetic modulation in vertebrates suggests that NMD is critical for cellular and tissue homeostasis, although the underlying mechanism remains elusive. Here, we generate knockout mice lacking Smg6/Est1, a key nuclease in NMD and a telomerase co-factor. While the complete loss of Smg6 causes mouse lethality at the blastocyst stage, inducible deletion of Smg6 is compatible with Embryonic Stem Cells (ESCs) proliferation despite the absence of telomere maintenance and functional NMD. Differentiation of Smg6-deficient ESCs is blocked due to sustained expression of pluripotency genes, normally silenced by NMD, and forced down-regulation of one such target, c-Myc, relieves the differentiation block. Smg6-null embryonic fibroblasts are viable as well, but are refractory to cellular reprograming into induced pluripotent stem cells (iPSCs). Finally, we see that depletion of all major NMD factors compromises ESC differentiation, thus identifying NMD as a licensing factor for the switch of cell identity in the process of stem cell differentiation and somatic cell reprogramming.

In addition, I would suggest to change the title back to 'Smg6/Est1 licenses embryonic stem cell differentiation via nonsense-mediated mRNA decay' given that the main focus of the paper is on the NMD-role for Smg6.

Before we can proceed to officially accept your manuscript for publication I also have to ask you to address the following minor editorial points:

-> please upload RNAseq data to the appropriate database and provide the accession number in the manuscript (if necessary as a placeholder number, the final number can be added in proof if there is waiting time for the database)

-> please make sure that the nature of the error bars are indicated for all figures presenting statistical data. I also noticed a few instances where you indicate n=2 for figures displaying significance values. Could you please either make sure that the data is based on independent triplicates or present the two sets of data points as series with statistics?

Thanks again for submitting your manuscript to The EMBO Journal, I look forward to receiving the final revision.

Referee #1:

This revised manuscript has been nicely revised and is nearly acceptable for publication. I have only two remaining concerns.

First, the MS is still written quite poorly. Particular attention should be paid to the Abstract, which does not flow well at all. Perhaps EMBO has good copy editors?

Second, the evidence that c-myc is a direct NMD target remains underwhelming. For example, it is not clear why the authors say c-myc mRNA has a 3'UTR structure that may destabilize it. What is it about the c-myc 3' UTR structure that suggests this? Long 3' UTRs can trigger NMD in some circumstances; is that what the authors are saying? If so, how long? Also, the authors should compare reporter expression driven by the c-myc 3' UTR vs. a control (stable) 3' UTR like beta-globin. If the c-myc 3' UTR elicits NMD, it should confer much lower reporter expression than the control 3' UTR. And knockdown of NMD should increase c-myc 3' UTR-mediated reporter expression but not control 3' UTR-mediated reporter expression.

Overall, this is an intriguing study that will be of great interest to scientists in many fields, including the RNA, developmental biology, and stem cell fields. I strongly recommend acceptance and think the Editors should consider having a commentary accompany it.

Referee #3:

In their revised manuscript, Li and colleagues address all the referees' comments either through clarification or by providing additional experiments. The authors convincingly demonstrate an essential role for Smg6 in ESC differentiation. Importantly, the roles of Smg6 in ESC differentiation are strictly linked to its NMD function. The essential function of the NMD pathway for ESC differentiation is very striking and I recommend the revised manuscript for publication in EMBO Journal.

2nd Revision - authors' response 13 February 2015

Reply to Referee #1

Second, the evidence that c-myc is a direct NMD target remains underwhelming. For example, it is not clear why the authors say c-myc mRNA has a 3'UTR structure that may destabilize it. What is it about the c-myc 3' UTR structure that suggests this? Long 3' UTRs can trigger NMD in some circumstances; is that what the authors are saying? If so, how long? Also, the authors should compare reporter expression driven by the c-myc 3' UTR vs. a control (stable) 3' UTR like beta-globin. If the c-myc 3' UTR elicits NMD, it should confer much lower reporter expression than the control 3' UTR. And knockdown of NMD should increase c-myc 3' UTR-mediated reporter expression but not control 3' UTR-mediated reporter expression.

Previous studies have shown that the 3'UTR structure stabilizes c-*myc* mRNA (Jones and Cole, 1987, *Mol Cell Biol*; Bernstein et al. 1992, *Genes & Dev*; Yeilding et al. 1996, *Mol Cell Biol*). An artificial 3'UTR around 420 nts can efficiently trigger NMD (Singh et al. 2008, *Plos Biology*). The c-*myc* 3'UTR reporter assay (Fig 5G) uses 3'UTR of 442 nts (ENSMUST00000161976) and has been extensively used to study the mRNA stability. Our data (altogether Fig 5E, F, G) indicate that 3'UTR is one of the determinants for the c-*myc* mRNA stability in Smg6-NMD proficient cells. We have clarified this point in our new manuscript by adding aforementioned references (page 14, parag 1, line 2).

