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Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells

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Transaction Report:

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

1st Editorial Decision 10 January 2012

Thank you very much for submitting your manuscript on potential conservation of pluripotency determinants between planarian- and mammalian stem cells for consideration to The EMBO Journal editorial office.
Please find attached the comments from three scientists that assessed the technically excellent and comprehensive inventory of planarian neoblasts including its relatively detailed comparison to mammalian stem cells. Given the rather descriptive nature of the study that does not functionally address pluripotency, refs#1 and #3 are despite explicit interest, not confident with some of the statements referring to planarians as informative model system for stem cell biology. Some further validation however could derive from attempts to knockdown the putative POU and Sox2 like transcription factors. Regarding the described knockdown assay that supposedly leads to abrogation of regenerative potential, it would be desirable to distinguish whether indeed reduced regenerative capacity or simply a reduction of proliferation/differentiation potential has been measured. Finally, both referees demand moderation of some currently unsubstantiated claims and correct presentation of COP9, TAF, INO80, mediator complex as essential for hESC-identity rather than direct Oct4/Nanog-regulators are demanded from the expert scientists. With these points in mind, we are happy to offer submission of a revised version for final assessment to our editorial office.

Please allow to remind you that it is EMBO_J policy to allow a single round of revisions only and that the ultimate decision will depend on content and strength of an adequately modified manuscript. Please do not hesitate to contact me directly (preferably via E-Mail) in case of further questions.

I am very much looking forward to your revised paper.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The planarian stem cells, neoblasts are known for their unprecedented regenerative capacity. These cells are beginning to be characterised in greater detail but much remains to be discovered about the mechanism that underpins this capacity for regeneration. In this study, neoblasts were purified and subjected to mRNA sequencing and shotgun proteomics, and the information obtained will primarily help towards further detailed studies on specific aspects in the future.

A number of questions and comments arise from this study:

1. A large number of epigenetic regulators detected in ESC are present in neoblasts. Since the targets of these regulators are unknown (also acknowledged by the authors in the last para of the Discussion), is it appropriate to claim 'deep conservation between mammalian and planarian stem cells"? If not, then the title of the paper is misleading. These factors are widely expressed within and between diverse organisms, but they work in a very context dependent manner. As the authors indicate, only future work on the targets of key epigenetic modifiers will show what their precise roles are.

2. This applies more with reference to OCT4, NANOG and SOX2 (Page 20-21). The authors attempt to make a case for a closer relationship between ESC and neoblasts, but do the authors consider that they have a strong case for this? Are the authors confident that unequivocal orthologs of these genes exists or will be found in planarians.

3. Studies on neoblasts per se are interesting and provide an understanding of an unusual stem cell. I consider that attempts by the authors to claim that neoblasts may be/or are like ESC is a bit forced and premature, which is not sustainable using available evidence. It is equally possible that they will in the end be considered interesting even if different from ESC, which might show how regeneration may have evolved separately while still using some basic components but with very different mechanistic solutions.

Leaving aside the points discussed above, I consider the work overall to be potentially important and technically sound, aand will serve as useful background information for future studies. Apart from the work showing that knockdown of some key genes affects regeneration in planarians, more in depth mechanistic studies will be needed in the future to gain greater knowledge of neoblasts, regardless of whether or not they resemble ESC.

Referee #2:

The authors dissociated planarian cells into single cells and sorted pluripotent stem cells (neoblasts) and differentiated cells by FACS, and then systematically conducted not only transcriptomic but also proteomic analyses using a second generation sequencer and LC-MS/MS, respectively. And then they comprehensively categorized the genes and their products using bioinformatics, and identified neoblast-specific or -enriched genes. They found that genes involved in (1) DNA replication and cell cycle regulation, (2) transcriptional regulation and chromatin organization, and (3) RNP-mediated post-transcriptional regulation, were strongly enriched in the neoblasts. However, these findings are just confirmation of previous findings, although they identified new genes and confirmed their function by RNAi. The most important or original finding of this study is that they found striking conservation of OCT4 and NANOG regulators in pluripotent stem cells during evolution. This is important because homologs of OCT4 and NANOG had not been identified or

confirmed in planarians previously, and their identification and expression shown here suggest that regulatory control of pluripotency is extremely old and perhaps even better conserved than the transcriptional regulatory relationships. In contrast, the targets of RBPs and epigenetic regulators have presumably changed extensively during evolution. These suggestions might be obtained only by this kind of systematic analysis. This paper is also well written with a variety of experiments. Thus I strongly recommend this paper to be published in EMBO journal.

However, before acceptance, I should ask the author to give some comments about the following point.

The authors introduce another example of this kind of gene network-evolution in the Introduction, but don't discuss this point in the Discussion.
"It should also be noted that even highly conserved regulatory networks can sometimes differ in key upstream transcription factors. For example, a key transcription factor that drives early embryonic development in Drosophila is Bicoid, while this factor is substituted by Orthodenticle and Hunchback in the beetle Tribolium"
Thus, I expect them to discuss this kind of "upside down" gene network-evolution in the Discussion (key regulator genes are usually conserved and downstream genes are diverse in general). Please summarize other examples of this kind of evolution and speculate why this type of evolution occurs during evolution of the pluripotent stem cell systems. I also expect them to identify the conserved elements of upstream regions of the homologous genes of OCT4 and NANOG regulators in the planarian genomes in the near future.

Referee #3:

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This work describes an extensive comparison of the transcriptome and proteome of planarian neoblasts with that of differentiated cells leading to the identification of about 4000 genes that were differentially expressed in neoblasts. A comparison with genes expressed in mammalian ES cells found a significant overlap and conservation between many of the genes expressed in neoblasts with genes known to be important for the maintenance of pluripotency in mammals. These genes include epigenetic regulators, targets of Oct4 and Nanog as well as regulators of the key pluripotency factors Oct4 and Nanog.

This paper describes a high quality and detailed analysis of gene expression in neoblasts and differentiated cells and is appropriate for publication in EMBO. However, the authors need to be more conservative and tone down their sweeping conclusions. The planarian system has major experimental limitations that need to be acknowledged and exaggerated conclusions such as "planarians are an informative model system for human stem cell biology" are totally unwarranted and need to be avoided. Here are a few examples where revisions are required.

1. The conclusion that the neoblast genes correspond to conserved "pluripotency" genes taken from the mammalian literature is based on the expression pattern in wt vs. irradiated animals and in impaired regeneration following knock down of candidate genes. At face value these criteria do not define these genes as being important for pluripotency but rather show that the observed phenotype could be caused merely by affecting proliferation of neoblasts. With other words, none of these assays give any relevant information on pluripotency. Thus, in contrast to mammalian ES cells where differentiation potential and self-renewal of ES cells can be separated, this cannot be done in this experimental design and that these genes have a similar function as in ES cells remains a hypothesis.

2. The key mammalian pluripotency genes Oct4 and Nanog were found to be not conserved in planarians. However, the authors conclude on page 20 that regulators of OCT4 and NANOG are conserved including "post-transcriptional regulation and chromatin remodeling complexes, such as spliceosome components, COP9, TAF, INO80 and Mediator complexes". This is a misinterpretation of the literature. While the knock down experiments (for example Chia et al, 2010) have identified these genes as being important for pluripotency of ES cells, no evidence identifies these genes as

"regulators of Oct4 and Nanog" which would imply that these complexes are upstream of these pluripotency genes. Thus, the argument that, although Oct4 and Nanog are not conserved but their "regulators" are which would support the identified genes as having a similar function as in ES cells, is not a valid one.

3. Similarly, the data in Figure 5C are interpreted to show that germ cell like posttranscriptional regulation is a "key factor in maintaining pluripotency in planarians". Again, the experimental evidence merely shows that these genes are expressed in neoblasts and this conclusion is not acceptable.

1st Revision - Authors' Response 29 March 2012

Response to the referees

We would like to thank the three referees for carefully reviewing our manuscript. We were happy to see that our work was considered as a "high quality and detailed analysis of gene expression in neoblasts" that is "potentially important and technically sound". It was pointed out that our insights "will serve as useful background information for future studies". The referees acknowledge the impact of our findings, for instance, that we found "striking conservation of OCT4 and NANOG regulators" and conclude that our "suggestions might be obtained only by this kind of systematic analysis" and finally that our study "is appropriate for publication in EMBO" (and that another referee "strongly recommends this paper to be published in EMBO journal").

We also appreciate the constructive critique that was raised by the referees. In what follows, we address all aspects that required correction or clarification.

Summary of important changes in the revision:

1. We performed additional experiments to address the comment of referee #3, pointing out that, although we were able to demonstrate functional requirement of epigenetic regulators during regeneration, the observed phenotype "could be caused merely by affecting proliferation of neoblasts". We first conducted additional RNAi experiments to demonstrate a defect in tissue turnover of healthy animals upon knockdown of epigenetic factors (see new Supplementary Figure S4). To investigate whether the observed failure of regeneration and tissue turnover is solely explained by a lack of neoblast proliferation or maintenance, we now present three different experimental assays for altogether three epigenetic/chromatin regulators (BRG1, SMARCC2, CTR9) and two additional genes (SETD8, SSRP1): First, we performed H3P staining to quantify the number of mitotic cells. Second, we quantified transcript expression of stem cell markers by RT-qPCR and ISH. Third, we measured mitotic cell content by flow cytometric analyses. Our data demonstrate that for the majority of analyzed epigenetic regulators, a lack of neoblast proliferation or maintenance cannot explain the observed regeneration and tissue turnover defects. The results are presented in the two novel Figures 4 and 6, and the new Supplementary Figures S4 and S5.
2. In response to the criticism of referee #1, we performed an improved screen for planarian homologs of OCT4, SOX2 and NANOG using profile Hidden Markov Models for multiple species alignments to achieve maximum sensitivity. This search provided candidates with putative homology to OCT4 and SOX2 for future studies. The search strategy is summarized in a new paragraph of the Supplementary Information and our candidate homologs are presented in the new main Figure 9 as part of the Discussion.
3. To address the critique of referee #1 and #3, who commented on our conclusion of conserved pluripotency control between neoblasts and ESCs, we amended the Discussion. We carefully explain our observation of conserved gene expression and what this could mean for the conservation of the underlying regulatory interactions. We discuss experimental limitations of the planarian model system and suggest future experiments to investigate these questions. To be more precise, we changed the title of the manuscript from "Molecular determinants of pluripotency are deeply conserved between mammalian and planarian stem cells" to "Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells"

4. Based on the referees comments we rewrote several sections, improved figures, and shortened the main text to the allowed number of characters.

We believe that our additional experiments and computational analyses helped to substantially improve the quality of the paper and to exhaustively answer all requests made by the referees and in the letter from the editor.

Detailed point-by-point response

Referee #1:

The planarian stem cells, neoblasts are known for their unprecedented regenerative capacity. These cells are beginning to be characterised in greater detail but much remains to be discovered about the mechanism that underpins this capacity for regeneration. In this study, neoblasts were purified and subjected to mRNA sequencing and shotgun proteomics, and the information obtained will primarily help towards further detailed studies on specific aspects in the future.

A number of questions and comments arise from this study:

1. A large number of epigenetic regulators detected in ESC are present in neoblasts. Since the targets of these regulators are unknown (also acknowledged by the authors in the last para of the Discussion), is it appropriate to claim 'deep conservation between mammalian and planarian stem cells"? If not, then the title of the paper is misleading. These factors are widely expressed within and between diverse organisms, but they work in a very context dependent manner. As the authors indicate, only future work on the targets of key epigenetic modifiers will show what their precise roles are.

Response: We agree with the referee that the enrichment of epigenetic regulators in ESCs alone does not provide sufficient evidence for the claim of "deep conservation between mammalian and planarian stem cells".

We concluded that enriched expression of epigenetic regulators is consistent with the known hyper-dynamic chromatin state of stem cells (see Discussion). Notably, the enrichment of orthologs of BAF, PcG, MLL and PAF complex components provides evidence for a specific similarity to ESCs, since these complexes are known to be important for maintaining ESC identity (see Results). We also demonstrated functional requirement during regeneration for BAF and PAF complex components and for the revised version performed now additional experiments to rule out a mere proliferation defect as an explanation for the observed failure of regeneration and tissue turnover (see summary of changes and novel figures 4 & 6).

Further supporting evidence for evolutionary conservation between ESCs and neoblasts comes from the observation that expression of genes associated with pluripotency control in human and mouse is conserved in neoblasts. In particular, we observed conservation of gene expression for direct mammalian targets of Oct4, Sox2 and Nanog, which are factors required for inducing and/or maintaining the pluripotent state of ESCs. Nonetheless, we agree that the title could be misinterpreted and suggest conservation of regulatory interactions, which we did not analyse.

Hence we decided to change the title from:

"Molecular determinants of pluripotency are deeply conserved between mammalian and planarian stem cells"

to:

"Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells"

Additionally, in the introduction we deleted the sentence

"Our results suggest overall deep evolutionary conservation of the molecular network regulating pluripotency in stem cells."

2. This applies more with reference to OCT4, NANOG and SOX2 (Page 20-21). The authors attempt to make a case for a closer relationship between ESC and neoblasts, but do the authors consider that they have a strong case for this? Are the authors confident that unequivocal orthologs of these genes exists or will be found in planarians.

Response: We would first like to point out that we applied state-of-the-art methods for the identification of orthologs. These methods have been successfully used in other well studied organisms such as Drosophila. We identified thousands of orthologs and validated some of them, in particular the ones discussed in more detail, by manually comparing domains and gene structure. For certain genes, however, orthology annotation is complicated by the fact they are members of gene groups with slightly different domains and functions of the members. Oct4 belongs to the group of POU domain proteins and this group comprises different classes of POU domains (POU1-POU6) that are in some cases very similar in sequence. Oct4 itself is a POU5 domain protein. Thus, to unambiguously determine domain orthology is highly non-trivial.

To address the reviewer's concern we applied a highly sensitive screening method for the identification of putative OCT4, SOX2 and NANOG homologs (see new paragraph "Screen for planarian homologs of human NANOG, OCT4 and SOX2" in Supplementary Information and Discussion). In short, we inferred a profile HMM from multiple species alignments of these factors and aligned the planarian proteome to these profiles. The highest ranking candidates were then carefully analyzed by hand. This way, we confirmed that no NANOG homolog could be identified in our transcriptome assembly, but we characterize in more detail putative homologs of OCT4 and SOX2. We decided to present putative OCT4 homologs in a new figure (Figure 9). We now present these candidates in the Discussion.

Having said this, our claim of conservation of pluripotency control applies not necessarily to these central pluripotency regulators but more generally to the expression of genes enriched in ESCs and ICM. Among the genes with conserved expression between ESCs and neoblasts are known direct targets of Oct4, Sox2 and Nanog and in the edited version we state explicitly that a large fraction of targets (70%-80%) displays conserved up-regulation in neoblasts (see Results). This observation indicates that the overall expression read-outs of ESCs and neoblasts are similar.

However, we are aware that our data do not allow for the conclusion that the regulatory *interactions* underlying the observed expression profiles are conserved between mammals and planaria. An alternative scenario could be the replacement of central regulators during evolution with conserved expression of target genes (see Discussion). For clarification and in order to provide an improved presentation of our conclusions, we re-wrote the last discussion paragraph.

3. Studies on neoblasts per se are interesting and provide an understanding of an unusual stem cell. I consider that attempts by the authors to claim that neoblasts may be/or are like ESC is a bit forced and premature, which is not sustainable using available evidence. It is equally possible that they will in the end be considered interesting even if different from ESC, which might show how regeneration may have evolved separately while still using some basic components but with very different mechanistic solutions.

Response: The data presented in our work indicate that many genes with a crucial function in ESCs are also over-expressed in neoblasts and for a number of these genes we could demonstrate a functional requirement during planarian regeneration. Since a large number of genes display conserved expression, we consider it unlikely that the entire underlying regulatory interactions have been rewired during evolution. On the other hand, we agree with the referee that aspects of regeneration might have evolved separately in planarians and mammals. Therefore, we do not claim that neoblasts are like ESCs, yet we conclude that neoblasts can be an informative model system for ESC biology, revealing both conserved and diverged mechanisms of regeneration (cf. comment 2.). To address the criticism, we re-wrote the last discussion paragraph and provide an improved presentation of our conclusions.

Leaving aside the points discussed above, I consider the work overall to be potentially important and technically sound, and will serve as useful background information for future studies. Apart from the work showing that knockdown of some key genes affects regeneration in planarians, more in depth mechanistic studies will be needed in the future to gain greater knowledge of neoblasts, regardless of whether or not they resemble ESC.

Referee #2:

The authors dissociated planarian cells into single cells and sorted pluripotent stem cells (neoblasts) and differentiated cells by FACS, and then systematically conducted not only

transcriptomic but also proteomic analyses using a second generation sequencer and LC-MS/MS, respectively. And then they comprehensively categorized the genes and their products using bioinformatics, and identified neoblast-specific or -enriched genes. They found that genes involved in (1) DNA replication and cell cycle regulation, (2) transcriptional regulation and chromatin organization, and (3) RNP-mediated post-transcriptional regulation, were strongly enriched in the neoblasts. However, these findings are just confirmation of previous findings, although they identified new genes and confirmed their function by RNAi. The most important or original finding of this study is that they found striking conservation of OCT4 and NANOG regulators in pluripotent stem cells during evolution. This is important because homologs of OCT4 and NANOG had not been identified or confirmed in planarians previously, and their identification and expression shown here suggest that regulatory control of pluripotency is extremely old and perhaps even better conserved than the transcriptional regulatory relationships. In contrast, the targets of RBPs and epigenetic regulators have presumably changed extensively during evolution. These suggestions might be obtained only by this kind of systematic analysis. This paper is also well written with a variety of experiments. Thus I strongly recommend this paper to be published in EMBO journal.

However, before acceptance, I should ask the author to give some comments about the following point.

The authors introduce another example of this kind of gene network-evolution in the Introduction, but don't discuss this point in the Discussion.

"It should also be noted that even highly conserved regulatory networks can sometimes differ in key upstream transcription factors. For example, a key transcription factor that drives early embryonic development in Drosophila is Bicoid, while this factor is substituted by Orthodenticle and Hunchback in the beetle Tribolium"

Thus, I expect them to discuss this kind of "upside down" gene network-evolution in the Discussion (key regulator genes are usually conserved and downstream genes are diverse in general). Please summarize other examples of this kind of evolution and speculate why this type of evolution occurs during evolution of the pluripotent stem cell systems.

Response: We present an additional example for plasticity of gene regulatory networks (for reasons of space limitations it was included as “Supplementary Discussion”) and discuss the possibility of similar scenarios in pluripotency control:

“Another example of this kind is the regulator of the transcription factor *otx,* which is crucial for establishing the regulatory state of the endomesoderm in two divergent echinoderms, the sea urchin *Strongylocentrotus purpuratus* and the sea star *Asterina miniata* (Hinman *et al*, 2007). The transcription factor *tbrain* is co-expressed with and required for expression of *otx* in the sea star and this regulatory role has been lost in the sea urchin while the downstream core regulatory network is conserved (Hinman *et al*, 2007). Gene networks of pluripotency control could have undergone similar changes during evolution, which, for instance, could have led to the inclusion of Nanog or other pluripotency regulators in the vertebrate lineage without changing the expression of downstream genes.”

I also expect them to identify the conserved elements of upstream regions of the homologous genes of OCT4 and NANOG regulators in the planarian genomes in the near future.

Response: We agree that characterization of targets and regulators of OCT4, SOX2 and NANOG is the next step to elucidate conservation of the pluripotency control gene network. In fact, the referee guessed part of the on-going work in our lab.

Referee #3:

This work describes an extensive comparison of the transcriptome and proteome of planarian neoblasts with that of differentiated cells leading to the identification of about 4000 genes that were differentially expressed in neoblasts. A comparison with genes expressed in mammalian ES cells found a significant overlap and conservation between many of the genes expressed in neoblasts with genes known to be important for the maintenance of pluripotency in mammals. These genes include epigenetic regulators, targets of Oct4 and Nanog as well as regulators of the key pluripotency factors Oct4 and Nanog.

This paper describes a high quality and detailed analysis of gene expression in neoblasts and differentiated cells and is appropriate for publication in EMBO. However, the authors need to be more conservative and tone down their sweeping conclusions. The planarian system has major experimental limitations that need to be acknowledged and exaggerated conclusions such as "planarians are an informative model system for human stem cell biology" are totally unwarranted and need to be avoided. Here are a few examples where revisions are required.

Response: We agree with the referee that the experimental limitations of the planarian system have to be discussed carefully and added the following paragraph to the discussion:

"Unfortunately, the use of planarians as a model organism for stem cell biology is currently still affected by certain experimental limitations: it is still unknown whether neoblasts represent a heterogeneous mixture of cells and which cells within this population are pluripotent. Moreover, it is currently impossible to culture these cells and perform controlled perturbation experiments. Finally, trans-genetics have not yet been established in planarians."

We also discussed our conclusion that "planarians are an informative model system for human stem cell biology" and whether we should keep it or not. We believe that this statement lives from the interpretation provided in the paper. It is based on our finding of overall conserved expression of genes with known function in ESCs and known requirement for the pluripotent state of these cells across human/mouse and planaria. For some of these genes we could also demonstrate functional requirement in planarian regeneration and based on additional cell proliferation assays we can rule out a proliferation defect as a sole explanation for the observed phenotypes. In response to this criticism, we re-wrote the discussion (specifically the last paragraph) to carefully express our hypothesis of conserved pluripotency control and clarify in what sense we consider planarian neoblasts as an informative model system for human stem cells. Therefore we would like to keep the statement. However, we propose to leave the final decision to the editor.

1. The conclusion that the neoblast genes correspond to conserved "pluripotency" genes taken from the mammalian literature is based on the expression pattern in wt vs. irradiated animals and in impaired regeneration following knock down of candidate genes. At face value these criteria do not define these genes as being important for pluripotency but rather show that the observed phenotype could be caused merely by affecting proliferation of neoblasts. With other words, none of these assays give any relevant information on pluripotency. Thus, in contrast to mammalian ES cells where differentiation potential and self-renewal of ES cells can be separated, this cannot be done in this experimental design and that these genes have a similar function as in ES cells remains a hypothesis.

Response: To understand whether the observed defects were trivially explained by a lack of neoblast proliferation or maintenance, we performed additional experiments for altogether five genes for which we had observed regeneration defects upon RNAi knockdown (see summary of changes). First, we performed H3P staining to quantify the number of mitotic cells. Second, we quantified transcript expression of stem cell markers by RT-qPCR and ISH. Third, we measured mitotic cell content by flow cytometry. Our data provide strong evidence that BAF complex components and SETD8 are required for differentiation and do not have non-redundant functions in neoblast maintenance and proliferation. For the analyzed PAF complex component, the observed moderate reduction of proliferating neoblasts is unlikely to fully explain the phenotype, and thus this factor is also likely to be involved in differentiation. We included additional figures into the manuscripts to present these new results: new Figures 4, 6 and Supplementary Figure S4, S5.

2. The key mammalian pluripotency genes Oct4 and Nanog were found to be not conserved in planarians. However, the authors conclude on page 20 that regulators of OCT4 and NANOG are conserved including "post-transcriptional regulation and chromatin remodeling complexes, such as spliceosome components, COP9, TAF, INO80 and Mediator complexes". This is a misinterpretation of the literature. While the knock down experiments (for example Chia et al, 2010) have identified these genes as being important for pluripotency of ES cells, no evidence identifies these genes as "regulators of Oct4 and Nanog" which would imply that these complexes are upstream of these pluripotency genes. Thus, the argument that, although Oct4 and Nanog are not conserved but their "regulators" are which would support the identified genes as having a similar function as in ES cells, is not a valid one.

Response: In their paper, Chia et al identify candidate pluripotency genes by using an RNAi screen in ESCs monitored by a POU5F1-driven GFP reporter construct. They further reduce this set of candidate pluripotency regulators by requiring down-regulation of endogenous OCT4 and NANOG

upon RNAi mediated knockdown of these candidates in three different human ESC lines. Chia et al never claim that all of the identified candidates are direct regulators of OCT4 or NANOG. However, one of the two analyzed candidates was shown to directly activate an OCT4 promoter in a reporter assay. Most of the other regulators will presumably only indirectly affect OCT4 or NANOG expression.

To clarify, this matter, we now introduce these genes as "direct or indirect regulators of OCT4 and NANOG expression in human ESCs" (see paragraph "Homologs of genes affecting OCT4 and NANOG expression in human ESCs are over-expressed in neoblasts" in the Results).

We also examined the function of the OCT4 and NANOG regulators for which we could identify planarian homologs. Only two epigenetic regulators (SUV39H2, HCFC1) were found among these genes. Therefore, the observed neoblast enrichment is not explained by an over-representation of this class of genes. Instead, proteins of the basal transcriptional machinery (TAF7, EIF2S2, EIF2B4, EDF1), several nucleoporins (TPR, NUP107) and post-transcriptional regulators (PCF11, NCBP1, SF3A1, SF3A3, SFRS3) appear in this gene set. Notably, genes associated with proliferation or survival of ESCs are not found among the planarian homologs of OCT4 and NANOG regulators.

To address the referee's concern we replaced the sentence

"Regulators of OCT4 and NANOG include components of transcriptional as well as post-transcriptional regulation and chromatin remodelling complexes, such as spliceosome components, COP9, TAF, INO80 and Mediator complexes."

in the Results section by

"Genes required for expression of OCT4 and NANOG with identified homologs in planaria include components of the basal transcriptional machinery (e. g. TAF7, EIF2S2, EIF2B4, EDF1), several nucleoporins (e. g. TPR, NUP107), post-transcriptional regulators (e. g. PCF11, NCBP1) and among those, in particular, splicing factors (SF3A1 and SF3A3)."

3. Similarly, the data in Figure 5C are interpreted to show that germ cell like posttranscriptional regulation is a "key factor in maintaining pluripotency in planarians". Again, the experimental evidence merely shows that these genes are expressed in neoblasts and this conclusion is not acceptable.

Response: We agree that the data shown in Figure 7 (old Figure 5) cannot be interpreted to conclude that post-transcriptional regulators represent key factors of neoblast pluripotency. However, previous studies, in particular from Agata's laboratory (Rouhana *et al*, 2010) have shown that knockdowns of neoblast enriched RBPs lead to depletion of neoblasts and/or differentiation defects. Thus, to clear up any confusion, we changed

"In summary, our genome wide profiling data concur with the accumulating evidences that germ cell-like post-transcriptional regulation is a key factor in maintaining pluripotency in planarian stem cells."

to

"In summary, our genome wide profiling data concur and broaden previous findings demonstrating the enrichment of post-transcriptional regulators in neoblasts and their contribution to the maintenance of stem cell identity (Rouhana *et al*, 2010)."