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DPY30 Regulates Pathways in Cellular Senescenece through ID Protein Expression

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

Editor: Thomas Schwarz-Romond

1st Editorial Decision 24 January 2013

Thanks you very much for submitting your study on DPY30 in regulating cellular senescence for consideration to The EMBO Journal editorial office.
Once more, apologies for the slight delay in providing comments, still caused by the recent holiday season.
You will easily recognize that the referees are in principle supportive of the data. They do provide however very constructive comments to broaden the scope by assessing more epigenetic marks (point 1 ref#1). This very relevant point would also strengthen the proposed mechanism that ref#3 judges as currently quite weak.
It would further be essential to address potentially conflicting results re SASP-factor expression (point #2, ref#1), expand the set of senescence marker (point 4, ref#1) and indeed aim for some physiological significance of this pathway (point 3, ref#1), preferably in a way/the system you eluted to in our previous correspondence?!

These are significant demands that certainly require further experimentation. I am however quite sure that you are in a rather strong position to expand/develop the study along the constructive comments from predominantly refs#1 and #3.

Please do not hesitate to get in touch (preferably via E-mail) in case you require further

clarifications OR to discuss a timeline for necessary amendments.

I very much look forward to receiving a suitably revised paper and hope that precise communication of essential demands for a more general title such as The EMBO Journal will facilitate efficient proceeds for your potentially interesting discoveries.

REFEREE REPORTS:

Referee #1:

Both transcription factors and epigenetic mechanisms play key roles in the implementation of senescent phenotypes, but very little is known about how these two mechanisms are integrated to establish senescence-associated gene expression. In this paper, Simboeck and co-workers reported that depletion of DPY30, a structural component of Set1/MLL complexes, causes induction of the p16^{INK4a} gene expression with the reduction of H3K4me3 level, an epigenetic mark for gene activation, around the p16^{INK4a} gene promoter. Interestingly, moreover, they found that ID genes are direct targets of DPY30 and depletion of DPY30 results in the reduction of ID gene expression, thereby causing activation of Ets1/2 transcription factors, leading to the activation of p16^{INK4a} gene transcription and consequent senescence-like cell cycle arrest. These finding are novel and provide a new insight into how senescence-associated gene expression is established.

In general, the experiments are well organized and data are for the most part solid.
However, there are several limitations which the authors should address before publication.

Critiques:

(1) The authors showed that the depletion of DPY30 causes induction of p16^{INK4a} gene expression with a decrease, but not an increase, of H3K4me3, an epigenetic mark for gene expression, around the p16^{INK4a} gene promoter. What is the status of repressive histone marks under these conditions , such as H3K27me3 and H3K9me2 (see Bracken et al., Genes Dev., 21: 525-530, 2007; Kotake et al., Genes Dev., 21: 49-54, 2007; Yamakoshi et al., JCB 186: 393-407, 2009; Wang et al., EMBO J. 32: 45-59, 2013)? I think the study would be more interesting if the authors can address this point.

(2) In Figure 3, the authors showed that the levels of SASP factor gene expression is reduced in replicative senescent cells. However, previous reports showed that the levels of SASP factor gene expression is increased in not only Ras-induced senescent cell but also in replicative senescent cells (see Figure 1E in Coppe et al., PLoS Biol., 6: 2853-2868, 2008). The authors should resolve this conflict.

(3) It is unclear when and where the pathway described here plays a role in vivo? I feel that the biological significance of this novel pathway remains unclear.

(4) The authors used SAHF as a marker of cellular senescence. However, recent reports have revealed that SAHF is dispensable for cellular senescence and question the role of SAHF in senescence cell cycle arrest (Kosar et al., Cell Cycle 10,3: 457-468, 2011; Di Micco et al., Nat. Cell Biol. 13: 292-302, 2011). Thus, the authors should use more reliable senescence marker(s), such as DNA damage response (DDR) and/or elevated level of reactive oxygen species (ROS).

(5) Page11 line 11 has misspelled; " H3K3me3" should be "H3K4me3".

Referee #2:

This is a well presented manuscript reporting experiments of high technical quality and addressing a

an issue of wide general interest, namely the molecular mechanisms underlying senescence and the role of the MLL1 component DPY30. The description of the experiments proceeds in a logical fashion and the text and figures are clear. The Discussion is fair and clearly sets out the conclusions that can reasonably be drawn. My only significant criticism is that parts of the Results section are too long. Too much time is spent describing results that do not really advance the main story. This is particularly true of descriptions of the ChIP-seq and microarray expression data. For example, the paragraph beginning "Several cytokines..." (p.8) mentions selected genes of potential interest, but this is anecdotal evidence at best. When several hundred genes change, there a bound to be a few that are potentially interesting. The paragraph beginning "The DPY30 microarray..." is similarly overlong and can be shortened. The problem with such descriptions is not that they are wrong, or that the data itself is uninteresting, but that they draw attention away from the main story. The authors might also give a bit more attention to the relationship between the global fall in H3K4me3 levels in cells depleted in DPY30 and the events at specific promoters. At first sight it is not surprising that H3K4me3 is often reduced around TSSs in depleted cells. The regions where H3K4me3 does not fall are perhaps more interesting.

Referee #3:

The manuscript by Simboek et al. describes a role of DPY30, a component of the Set1 and MLL complexes, in the control of cellular senescence. They show that depletion of DPY30 expression induces senescence with up-regulation of CDKIs in NT2 and IMR90 cells. The up-regulation of CDKIs occurs even though H3K4me3 is lost from their promoters, thus their up-regulation is H3K4me3-independent. Using both expression arrays and ChIP-seq data, they identify IDs as DPY30 targets, and show that ectopic ID1 or ID3 expression can partially rescue the senescence phenotype induced by DPY30 depletion.

This work is potentially interesting, but the suggested mechanism of how shDPY30 induces senescence is quite weak. It is well known that p16 expression is also regulated via H3K27me3 (e.g. PcG and H3K27me3 demethylase) during senescence, and I wonder if the authors have looked at this histone mark. Can the potential involvement of the H3K27me3 axis explain why the ectopic IDs show only a weak effect?

Fig. 4D: the authors assume that the 600 downregulated genes with less H3K4me3 in shDPY30 cells are direct DPY30 targets. However, since not all H3K4me3 genes are DPY30 positive in control cells (Fig. 4A), they have to overlay this gene set (600 genes) with DPY30-positive genes in control cells (3587 genes shown in Fig. 4A) to get the 'direct' targets.

I am confused by Fig. 4B. The label suggests that this is a Venn diagram for H3K4me3 in control and shDPY30 cells.... I may be missing something, but isn't it supposed to be a simple bar graph?

The ectopic expression of ID1 or ID3 leads to a bypass of shDPY30-induced senescence, but co-expression of E2F2 and ID1/ID3 doesn't. Can the authors comment on this? Also, can they rescue shDPY30-senescence by ID over-expression in NT2 cells as well?

The authors show that ectopic IDs partially rescue shDPY30-induced senescence/p16 up-regulation, but it seems to be a secondary effect, since it takes two to three weeks. Why does it take so long to reduce p16 levels after ID over-expression?

Minor points:

Fig.5D should include a representative panel from shCtrl cells.

I didn't find any mention in the main text of Sup. Fig 6B and Sup. Fig 6D.

1st Revision - authors' response 03 June 2013

Referee #1

"These finding are novel and provide a new insight into how senescence-associated gene expression is established. In general, the experiments are well organized and data are for the most part solid."

We thank the reviewer for his/her positive comments on our work.

Major points

1. The authors showed that the depletion of DPY30 causes induction of $p16^{INK4a}$ gene expression with a decrease, but not an increase, of H3K4me3, an epigenetic mark for gene expression, around the $p16^{INK4a}$ gene promoter. What is the status of repressive histone marks under these conditions , such as H3K27me3 and H3K9me2 (see Bracken et al., Genes Dev., 21: 525-530, 2007; Kotake et al., Genes Dev., 21: 49-54, 2007; Yamakoshi et al., JCB 186: 393-407, 2009; Wang et al., EMBO J. 32: 45-59, 2013)? I think the study would be more interesting if the authors can address this point.

As requested by this reviewer, we have now analyzed the H3K27me3 and H3K9me2 levels on the p16INK4a promoter in both control and DPY30-depleted IMR90 fibroblasts. Moreover, we have also performed this analysis in the presence of ectopically expressed ID1 and ID3.

Both repressive marks decreased in DPY30 knockdown cells, and this decrease nicely correlated with the transcriptional activation of p16INK4a (Fig. 5F). A similar decrease of H3K27me3 as well as EZH2 binding was observed in DPY30-depleted NT2 cells (Sup. Fig. 4C). Of note, partial rescue of p16INK4a repression by ectopic overexpression of ID1 or ID3 did not correlate with a re-establishment of repressive marks H3K27me3 or H3K9me2. We believe that this interesting observation suggests that transcriptional control of this locus could also occur in a Polycomb-independent manner. As now mentioned in the Discussion section, this is likely mediated by the decreased binding of the ETS1/2 transcription factor (due to an increased ID protein expression). In addition, we identified EZH2 as a direct DPY30 and H3K4me3 target gene whose expression decreased 1.5-fold upon DPY30 depletion, resulting in decreased H3K27me3 deposition at the promoter. These findings have now been added to the Results and Discussion sections.

2. "In Figure 3, the authors showed that the levels of SASP factor gene expression is reduced in replicative senescent cells. However, previous reports showed that the levels of SASP factor gene expression is increased in not only Ras-induced senescent cell but also in

replicative senescent cells (see Figure 1E in Coppe et al., PLoS Biol., 6: 2853-2868, 2008). The authors should resolve this conflict."

Coppe *et al*. reported that several SASP factors are upregulated in replicative senescence similar to what is observed in OIS. In this study, the authors did not analyzed mRNA levels but rather used antibody arrays against 120 proteins to detect secreted factors from different sets of replicative senescent fibroblasts grown in 3% and 20% of oxygen.

In contrast, in our study, we aimed to investigate the **direct** effect of DPY30 (MLL/Set1) on transcriptional regulation. Therefore, we used a microarray data set of replicative senescent IMR90 cells grown under the conditions (20% of oxygen) used in our experiments (GSE19018). It is well established that mRNA deregulation does not always overlap with the level of translated protein being secreted. In addition, several SASP factors, such as IL6, IL8 and ICAM, are in fact not highly secreted in senescent IMR90 when grown under 20% of oxygen (see Coppe et al., Plos Biol., 6: 2853-2868, 2008, Figure 1). This is actually consistent with the expression data obtained from the microarray GSE19018.

Since we performed our study using IMR90 fibroblasts grown under 20% of oxygen, we originally compared our DPY30 knockdown expression data to senescent IMR90 grown under the same condition. Considering this referee's comment, we have now also compared the DPY30 knockdown expression data to the data set of replicative senescent IMR90 cells grown under 3% of oxygen (Sup. Fig. 7 and 8). Interestingly the overlap of deregulated genes in replicative senescent cells grown under 3% versus 20% is very low. Considering stringent criteria of a *P* value smaller than 0.05 and a 2-fold change in expression, we could only identify the SASP factors IL8 and IL1B as transcriptionally upregulated in the 3% oxygen condition. Therefore, we do not believe that there is a discrepancy between our finding that SASP factor gene expression is reduced in DPY30 knockdown cells and earlier studies on the role of SASP in replicative senescent cells.

3. "It is unclear when and where the pathway described here plays a role in vivo? I feel that the biological significance of this novel pathway remains unclear."

Although we believe that our data linking ID proteins and DPY30 with cell proliferation provide an important biological significance, we have further investigated their role in aging, using epidermis as a model system.

RNA sequencing of epidermal stem cells isolated from young (3 month-old) and old (18 month-old) mice revealed a decrease of DPY30 expression in aged mice

(Dole et al., 2012). This nicely correlated with the downregulation of ID1. We confirmed that DPY30 and ID1 are expressed (as shown by RT-PCR) in epidermal stem cells isolated from young (3 month-old) and old (18 month-old) mice.

In addition, we monitored DPY30 and ID protein expression in keratinocytes of young (3 month-old) and old (20 month-old) mice and observed a robust downregulation (Sup. Fig. 12A). Immunofluorescence against ID1 of epidermal sections of young and old mice revealed that it is downregulated in the skin of the old mice (Sup. Fig. 12B). However, neither the DPY30 antibody produced in our laboratory nor the commercially-available DPY30 antibody (Santa Cruz sc-167677) specifically recognized mouse DPY30.

Furthermore, DPY30 expression was also reduced in replicative senescent IMR90 fibroblasts, similar to aged epidermal stem cells and keratinocytes. (Sup. Fig. 12C). Reduced ID proteins expression in replicative senescence has already been reported (Zebedee and Hara, 2001), but it was unclear how ID protein expression is regulated during aging.

Thus, we provide direct evidence that DPY30 (MLL/Set1) regulates ID protein expression: decreased expression of DPY30 during aging resulted in lower ID protein levels, leading to enhanced p16INK4a expression.

4. "The authors used SAHF as a marker of cellular senescence. However, recent reports have revealed that SAHF is dispensable for cellular senescence and question the role of SAHF in senescence cell cycle arrest (Kosar et al., Cell Cycle 10,3: 457-468, 2011; Di Micco et al., Nat. Cell Biol. 13: 292-302, 2011). Thus, the authors should use more reliable senescence marker(s), such as DNA damage response (DDR) and/or elevated level of reactive oxygen species (ROS)."

We agree with referee #1, and we have performed additional analyses of other senescent markers, including DDR and ROS.

While immunofluorescence for γH2A.X in DPY30-depleted NT2 cells clearly indicated DNA breaks (Sup. Fig. 1C), Western blot (Fig. 1B) and immunoflourescence (Sup. Fig. 2) analyses for γH2A.X in DPY30-depleted IMR90 cells resulted in only a mild increase as compared to RAS-induced senescence in the same cell type. However, shDPY30 IMR90 cells clearly showed foci stained for TP53, another marker for DNA double-strand breaks (Sup. Fig. 2). In addition, the level of reactive oxygen species (ROS) was significantly increased in shDPY30 cells, similar to that in RAS-induced senescent cells (Fig. 1C).

As all tested senescence markers (including SA-β-galactositase, increased p16INK4a expression, SAHFs and DDR and ROS) were positive in shDPY30 cells, we feel confident in claiming that loss of DPY30 induces a senescent-like phenotype. We thank the referee for suggesting these important experiments.

5. *"Page11 line 11 has misspelled; " H3K3me3" should be "H3K4me3"."*

We have fixed the corresponding text and apologize for the typos.

Referee #2

"This is a well presented manuscript reporting experiments of high technical quality and addressing a an issue of wide general interest"

We thank the reviewer for his/her positive comments on our work.

"My only significant criticism is that parts of the Results section are too long… The problem with such descriptions is not that they are wrong, or that the data itself is uninteresting, but that they draw attention away from the main story."

We agree with referee #2 and as requested have shortened these sections significantly. However, we would prefer to retain part of the description of target genes, since we want to underscore that the observed phenotype is only senescence-**like** and does not necessarily implicate the presence of the classic transcriptome of fully senescent cells (*e.g.* SASP factors). In addition, we consider that validation of the microarray and of the ChIPseq analyses to be important, and want to state briefly why we chose some genes (including ID proteins, which we later found to be key players in a senescence-like pathway regulated by DPY30) for validation.

"The authors might also give a bit more attention to the relationship between the global fall in H3K4me3 levels in cells depleted in DPY30 and the events at specific promoters. At first sight it is not surprising that H3K4me3 is often reduced around TSSs in depleted cells. The regions where H3K4me3 does not fall are perhaps more interesting."

In our analyses, we identified about 10,000 genes with unchanged H3K4me3 levels at three days after selection. We believe that most of these genes would undergo a loss of H3K4me3 at a later time point. This is indeed supported by a severe loss of global H3K4me3 levels after six days of infection as compared to three-days post-infection. However, for ChIPseq and microarray analyses, we chose the earlier time point in order to focus on genes that are likely implicated in establishing the observed senescence-like phenotype. Indeed, genes with unchanged H3K4me3 levels might also be involved in the observed phenotype; however, we could not draw any clear conclusion of whether these 10,000 genes are involved in various molecular processes. In addition, we could not detect any good overlap with upregulated genes, which made it difficult for us to focus on these genes.

Referee #3

1. "It is well known that p16 expression is also regulated via H3K27me3 (e.g. PcG and H3K27me3 demethylase) during senescence, and I wonder if the authors have looked at this histone mark. Can the potential involvement of the H3K27me3 axis explain why the ectopic IDs show only a weak effect?"

We monitored levels of H3K4me3, H3K27me3 and H3K9me2 in DPY30 knockdown cells both in the absence and presence of ectopically-expressed ID1 and ID3 (Fig. 5F). Upon activation of p16INK4a in DPY30 knockdown cells, the repressive histone marks H3K27me3 and H3K9me2 were lowered. Interestingly, in cells overexpressing the ID protein, H3K27me3 and H3K9me2 levels remained low, which could explain why the phenotype and p16INK4a re-repression are only partially rescued, as indeed suggested by referee #3.

Interestingly, we found that EZH2 is a DPY30 (and H3K4me3) target gene. The expression of EZH2 is 1.5-fold downregulated in DPY30 knockdown cells. This could explain why p16INK4 expression cannot be fully re-repressed in DPY30 knockdown cells. We now included this important point in the Discussion section.

2. "Fig. 4D: the authors assume that the 600 downregulated genes with less H3K4me3 in shDPY30 cells are direct DPY30 targets. However, since not all H3K4me3 genes are DPY30 positive in control cells (Fig. 4A), they have to overlay this gene set (600 genes) with DPY30-positive genes in control cells (3587 genes shown in Fig. 4A) to get the 'direct' targets."

In the previous version of our manuscript, we overlapped the downregulated genes with genes that display a decrease in H3K4me3, since *i)* the DPY30-MLL/Set1 complex does not necessary remain bound to promoters after depositing the H3K4me3 mark (therefore we believed that changes in H3K4me3 levels are a better readout for a direct consequence of DPY30 loss on the transcription of target genes); and *ii)* we believe that in the DPY30 ChIPseq, not all direct DPY30 targets were identified due to technical limitations (the DPY30 antibody is less efficient than the H3K4me3 antibody; crosslinking of DPY30 to DNA is less efficient than that of histones to DNA).

However, as suggested by referee #3, we now show that analyzing the overlap of downregulated genes, DPY30 target genes, and genes with less H3K4me3 in shDPY30 cells led us to identify 95 genes (Sup. Fig. 8E), including ID1, ID3 and E2F2.

3. "I am confused by Fig. 4B. The label suggests that this is a Venn diagram for H3K4me3 in control and shDPY30 cells…. I may be missing something, but isn't it supposed to be a simple bar graph?"

We apologize for the confusion. We have changed the Venn diagram in Fig. 4B to a pie chart, which we believe is more informative.

4. "The ectopic expression of ID1 or ID3 leads to a bypass of shDPY30-induced senescence, but co-expression of E2F2 and ID1/ID3 doesn't. Can the authors comment on this? Also, can they rescue shDPY30-senescence by ID over-expression in NT2 cells as well?"

The role of E2F transcription factors, and in particular E2F1, in senescence is controversial. In general, E2F1 positively regulates progression of the cell cycle by activating transcription of genes required for DNA synthesis. Accordingly, E2F1 was shown to be downregulated in senescent fibroblasts (reviewed in Campisi et al., 1996). Interestingly, tumor cells can be driven into a senescence-like cell state by depleting E2F1, and this occurs independently of p53 and pRb. Ectopic expression of E2F1 in these cells bypasses cellular senescence (Park et al., 2006). Other studies however have shown that ectopic expression of E2F in human fibroblasts induces senescence, by a direct activation of the p14ARF tumor suppressor gene. Fibroblasts deficient for p53 or p14ARF are immune to E2F1-induced senescence (Dimri et al., 1999).

Neither finding is surprising given the nature of E2F1. On the one hand, as a major regulator of cell cycle progression, E2F1 expression could lead to a senescence bypass. On the other hand, E2F1, like activated Ras or Raf, is potentially oncogenic when highly expressed, which could lead to OIS to prevent tumorigenesis. Thus, it was suggested that the impact of E2F overexpression on senescence is somehow dose-dependent. Indeed, in our cell system, we observed a strong overexpression of E2F2, which in fact might direct the cells more towards senescence, even in presence of ectopic ID protein expression. Therefore we suggest that in this setting (*e.g.* high E2F2 overexpression) ID protein overexpression cannot rescue senescence induced by the loss of DPY30. We now discuss this interesting possibility.

Whether ID protein overexpression in DPY30-depleted NT2 cells could also (at least partially) rescue the senescent-like phenotype is an interesting point from referee #3. As requested, we performed this experiment (multiple times). However, we could not overexpress ID proteins in NT2 cells above endogenous levels. In IMR90 cells, we first infected (retrovirus) cells to overexpress ID proteins, and then performed a second infection (lentivirus) to knockdown DPY30.

We applied the same strategy for NT2 cells, but could not detect any ectopic overexpression of ID proteins.

We have tested in several setups different virus concentrations for infections, which resulted in efficient infection of both 293T and NT2 cells. However, ectopic overexpression of ID proteins was only achieved in 293T cells, similar to IMR90 cells, but not in NT2 cells. We believe that ectopic overexpression of ID1 and ID3 is not possible in NT2 cells since endogenous expression levels are already very high. We reasoned that we could change the order of infections and first knock-down DPY30, which would results in decreased ID protein expression. However, upon knockdown of DPY30, cells stopped dividing and entered a senescence-like state, making this approach of a second retroviral infection impossible.

In addition to NT2 cells, we have tested the effect of DPY30 knock-down in various cancer cell lines: breast cancer cells (T47D and MCF7), prostate cancer cells (PC3), and leukemic cells (NB4 and U937) (unpublished observations). As in NT2 cells, MCF7, PC3 and NB4 cells displayed a high level of endogenous ID protein expression. High expression of ID proteins in cancers is actually considered to be a tumor marker. Upon DPY30 knock-down, ID protein expression levels were strongly decreased, and cells proliferate much less.

5. "The authors show that ectopic IDs partially rescue shDPY30-induced senescence/p16 up-regulation, but it seems to be a secondary effect, since it takes two to three weeks. Why does it take so long to reduce p16 levels after ID over-expression?"

Rescue or bypass of senescence in general is a very slow process and has, to our knowledge, never been published to occur in less than 14 days (e.g. Acosta et al, 2008; Rovillain et al, 2011). Therefore we believe that it is not surprising that the effects of the partial rescue that we observe upon ID overexpression become visible after 2-3 weeks. This is especially true as we believe that other factors that might contribute to a full-rescue of senescence have not been identified. We favor a model of rescue in which cell-to-cell fluctuations in the expression of those other genes important for bypass are increased in the background of ID proteins overexpression.

Additionally, and as already mentioned, we found that the Polycomb protein EZH2 is a DPY30 target. Its expression is decreased upon shDPY30 and most likely PRC2 complex activity is also affected. This is not restored when ID proteins are overexpressed. Consequently, p16INK4a is not fully re-silenced since the H3K27me3 mark at its promoter stays low (Fig 5F), leading only to a partial rescue. It is possible that if Polycomb action could be restored in DPY30

knockdown cells, the partial and delayed rescue by ID protein overexpression could be improved and accelerated.

Minor points:

"Fig.5D should include a representative panel from shCtrl cells."

Colony formation assay of knocked-down control cells has been included in Fig. 5D.

"I didn't find any mention in the main text of Sup. Fig 6B and Sup. Fig 6D."

This typo has been corrected.

2nd Editorial Decision 19 June 2013

Thank you very much for the revised study that was assessed by one of the original referees.

Before formal acceptance, please notice that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with such information. These will be linked online as supplementary "Source Data" files. Please do let me know if you have any questions regarding this initiative.

Further, the referee noted a small typo in the ms that should not be overlooked at the proof-stage.

Please allow me to congratulate you to the study. I look forward to receive relevant source data soon and assure you that the editorial office will be in touch with necessary paperwork related to official acceptance.

REFEREE REPORT:

Original referee #3

The authors have addressed most of my questions, and I am in support of publication of the manuscript.

Typos:
Page 10, the last sentence: 'RT-PCR' should be 'qPCR'.

