nature research

Peer Review File

Manuscript Title: Reprogramming to recover youthful epigenetic information and restore vision

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

This is very interesting and timely paper.

Why and how the young nervous system can regenerate better than the more mature, aged on can, if fundamental to neuroscience and medicine. I think very highly of the groups involved in this paper- they are true experts in their respective fields. There are some serious issues with the in vivo phenotypes and some of the wording in the text, however. These need resolution. I am not fan of cryptic reviews so I will make the points that I feel need new data, analysis and/or text changes as clear as possible. Again, a very interesting paper with great potential, provided the revisions are made.

Major issues:

1) In the introduction the authors state:

"To date, attempts to reverse acute or chronic damage to the CNS have been moderately successful, and no treatments have successfully restored eyesight."

This statement is false: more than a dozen papers now show some degree of recovery of axon growth (many from He lab are among the best in that collection) and several papers from Dr. He's lab have shown functional recovery, as has work from Benowitz lab (also at Harvard Med), and Lim et al., Nature Neuroscience (2016).

I can only speculate that Dr. He (listed as second to last author) didn't see the final text but this stands out as a major concern and sets the stage for the other 3 major issues:

2) The degree of optic nerve regeneration is modest compared to Park et al. 2008 Science, and work on PTEN/SOCS3 conditional deletions, as well as Lim et al., 2016 and de Lima, PNAS 2012 (Benowitz lab). The fibers shown in the main figures don't extend to the chiasm or beyond (an issue for the functional recovery data; an eye divorced from the targets can't restore function.
The supplemental data S4 show regrowth into the chiasm but regeneration accessory optic targets (MTN, DTN, NOT) are what is required to restore the functions examined here, both at the level of acuity and at all. 3 papers have shown that those targets and those targets only are the ones that mediate OKR/OKN in the mouse. They do only require a minimum of axons to drive those functions but they need *Some* axons from retina to get there. De-nervation of those targets and re-innervation of them after the treatments here, are necessary to conclude any degree of true functional recovery. The controls don't resolve this currently.

3.The glaucoma model involves gradual loss of RGCs. There are scores of IOVS and J Neuro papers showing some functional recovery in such models. The text here makes it seem as if the authors reached a key milestone. This is simply not the case. "To our knowledge, REVIVER is the first treatment to reverse vision loss in a glaucoma model" (No)

4.Ironically, while they vastly overstated the novelty of their work (see comments and references above) the authors gave up the chance to highlight what is arguably the most important and novel aspect of their findings- that they can perform the intervention (OSK) after nerve injury and see the greatest effects. Previous works (all those listed above in fact) had to be done prior to the injury... which implies limited therapeutic potential or at least creates a need for novel delivery platforms. The ability to

introduce a epigenetic or other manipulations to enhance regeneration of already injured CNS axons at any point across the Lifespan is exciting indeed. I would hope the authors would emphasize this point going forward.

Minor:

1. Space is limited but please explain Hutchison-Guilford Syndrome.

2. Ditto for Shannon Information Theory and aspects of which are relevant for stored copies of genetic info. I'm familiar with this but the mention of it in the context of a short paper (several times) without much meat or context, makes it confusing for the non-expert.

3. The authors should mention the Kennard Principle: the "negative linear relationship between age of a brain lesion and the outcome expectancy: in other words, that the earlier in life a brain lesion occurs, the more likely it is for some compensation mechanism to reverse at least some of the lesion's bad effects".

4. The display of the retina with RGCs at the top of the image will make Ophthalmologists pleased and neuroscientists confused, despite the location of RBPMs staining. This is not critical to my assessment at all, but consider flipping these to match the convention in 99% of the papers.

_____

Again, an exciting paper. Epigenomic control of regeneration capacity is fundamentally interesting. There are several technical gems here too: the use of the TET system, the AAVs themselves and the gymnastics the authors went through to get large sequences into them and expressed in vivo. These are not lost on me. The authors are to to be commended for the work.

Additional points:

There appears to be a dramatic decline in the number of axons regenerated between the Tet-On and Tet-Off system, i.e. Fig 1e and Fig. 2c. Please explain the discrepancies and how the system used might contribute to this difference.

The decline in DNA methylation age seen with human neuronal cultures needs to be controlled better to really establish the similarity in effects between mouse and human. While the cultures treated with OSK are much higher in age before VCS treatment (fig. 2h), the control cultures in extended Fig .8e are comparatively younger before VCS.
Further, in the control cultures, VCS treatment only seems to have a marginal effect on day 1, with some decline in age at day 9. With this in mind, please tone down the conclusions about how conserved the effects of OSK are.

--The authors have stated that the increase in visual acuity restores about half of vision loss...This is an exaggerated statement as they have only shown one behavior that is partially recovered. Further, in Fig 3f and h, they have not indicated if the recovery in visual acuity is significant between the saline and +OSK groups.

In the glaucoma model, the authors state that 'the axon regeneration is slightly less than young mice two weeks after injury (fig. 4b)'. Presumably they are talking about the Tet-Off group in Fig 1 and not the Tet-On group? Please clarify.

Referee #2 (Remarks to the Author):

In this manuscript, the authors examined the impact of Oct4/Sox/Klf4 (OSK) co-expression on retinal ganglion cell (RGC) axon regeneration and function. They provided evidence that sustained expression of OSK promote axon regeneration in the crush and glaucoma models as well as promote visal function in aged animals without axon regeneration. Such manipulates leads to changes in DNA methylation of a few age-related marks, which as well as axon regeneration requires functions of Tet1 and Tet2. The experiments appear to be well done and the results are of high quality.

The author started in the introduction by discussing reversal of aging. The authors then provided evidence showing that only when OSK are sustained expressed after injury axon regeneration can be induced, therefore this approach does not reprogram the RGCs to a youthful state as in development (which can regenerate axons), but instead promotes axon regeneration directly. A number of studies have already shown various ways to promote axon regeneration of RGCs and the concept of reprogramming to promote axon regeneration is not particular novel (e.g. PMID: 19815778; 28641110). DNA demethylation pathway and tet proteins have already been shown to be required for effective PNS and CNS axon regeneration in mice. Therefore, the conceptual advance of the current study is limited. On the other hand, the application of their system in the Glaucoma and aging models is new and interesting. There is not sufficient data to fully support the concept of "recovery of information via epigenetic reprogramming"/REVIVER".

Major concerns

The authors need to perform gene expression analysis of RGCs at different ages and upon OSK expression with or without injury. Does OSK expression leads to expression of regeneration associated genes in RGCs after injury? How about the DNA methylation status of these regeneration associated genes? The authors have the RRBS datasets in hand.

Given only 70% overlap of shRNA-Tet1 or Tet2 expression with OSK vectors, you would expect still see significant number of regenerating RGC axons. But the results showed no axons longer than 500 um (Fig. 2f).

The experiments with SH-SY5Y cell lines (a tumor cell line, developmental immature neurons) is not very relevant and should be removed (Fig. 2g-j).

In the glaucoma model, the authors need to show regeneration axons, instead of axonal densities. What's the infection rate for OSK expression in this model? The recovery is almost complete (Fig. 3d). Is the effect completely cell autonomous?

The authors should also perform optomotor response behavior test in the axon crush model for young and old animals.

The RNA-seq analysis of 5 months and 12 months old animals are interesting. Does the recovery of gene expression to levels of 5 months old animals by OSK expression require Tet1 or tet2? How about gene expression in response to injury at two different stages with or without OSK expression?

Other comments

Are all regenerated axons with OSK expression?

How long after injury can expression of OSK still promotes axon regeneration?

Does OSK expression lead to elevation of 5hmC levels in RGCs after injury?

Tet proteins are known to exhibit functions independent of active DNA demethylation. A confirmation of

role of active DNA demethylation requires other manipulations, such as downstream Tdg manipulation (PMID: 28426967).

Referee #3 (Remarks to the Author):

The manuscript from Lu and colleagues reports striking finding that in vivo expression of Yamanaka factors confers on RGCs axon regeneration capacity after injury, which is lost after birth. They further show that this axon regeneration leads to vision recovery in glaucoma model mice and in normal aged mice. These findings are of huge importance in that the extent of the axon extension and the vision recovery has been vastly improved compared to other studies as of today. Moreover, through their observation that the DNA methylation level correlates well with the regenerative capacity, they set out to examine the functional roles of Tet1/2 demethylases by introducing shRNAs against Tet1/2. Their results suggest that altered DNA methylation is associated with acquisition of the regenerative capacity by OSK. The current study sheds light on modification of DNA methylation as a new way to counteract age-related diseases and natural aging.

Major concerns:
1.
As the authors mention in the manuscript, it has been well established that the level of DNA methylation at particular locus is gradually increased along aging and that it works as an aging biomarker, also known as the DNA Methylation Clock. However, what the functional roles of the DNA Methylation Clock in aging are and whether reversing the clock can alleviate functional decline by aging are still unknown and, therefore, a subject many researchers put a ton of effort into.
The authors demonstrate that expressing the reprogramming factors can indeed decrease the DNA methylation level at the rDNA regions (rDNAme clock), which prompts us to assume that rDNAme might have a link to the acquisition of the regenerative capacity. However, given that tons of molecular events are provoked and multiple pathways are activated downstream of the OSK expression, I would have to say the experiments in the manuscript fail to successfully exclude the possibility that the reversal of the rDNA clock and the acquisition of the regenerative capacity are independent events and have no causal relationship.

2.
Having said that, the observation that downregulation of Tet1 or Tet2 suppresses the OSK-dependent youthful phenotypes strongly suggests that erasure of DNA methylation is involved in the process of "rejuvenation".
Considering Tet1 and Tet2 control DNA demethylation genome-widely, it is unlikely that alteration in DNA methylation can be seen only in rDNA regions. Therefore, the authors should analyze DNA methylation changes not only in the specific locations, e.g. rDNA loci, but also in an unbiased manner. An analysis of global DNA methylation profiles in a decent set of samples, such as -/+ OSK, -/+ Tet1/2 alone is invaluable, as it will certainly facilitate to understand the molecular mechanisms by which OSK confer regenerative capacity on RGCs.
When such DNA methylation analysis is performed, a sample in which Dox is withdrawn after OSK-mediated regeneration should be included, as OSK expressing cells presumably have molecular profiles far apart from normal RGCs with OSK being turned on.

3.
DNA demethylation occurs through an active pathway and a passive pathway, which are through cell division and by TET DNA demethylases, respectively. As such, it is critical in the current study to demonstrate that RGCs do not proliferate by the OSK introduction. The authors indeed show no upregulation in Ki67. However, this alone does not convincingly support the statement. The authors should conduct additional assays, such as BrdU incorporation in a quantitative way, to make the statement more convincing.
In Fig. 1f, the authors show the numbers of RGCs per mm2 16 hours after the nerve crush as survival rates. The authors should also examine time course changes in the numbers of RGCs after the crush to

confirm the lack of cell proliferation. This would give clearer interpretation since the positive control for the immunostaining in Extended Fig. 4a is not the best one, which is HEK293 cell line.

4.
Tet1 and Tet2 are known to be, at least in large part, functionally redundant. Thus downregulating only either of the two are more likely to have no or little effect on DNA methylation. This point should be addressed, or at least discussed in the manuscript.
On that note, the expression levels of Tet1 and Tet2 are not examined in the experiments using shRNAs against them. The authors need to add the data to show how much the suppression efficiency is. This will also provide the information how specific these shRNAs are in the RGC context. In addition, the expression levels of Tets in RGCs and those in RGCs expressing OSK should be examined.
Tet1 KO mice and Tet2 KO mice were previously generated (Dawlaty MM et al., Cell Stem Cell 2011). In addition, genomic locations where the DNA methylation level is upregulated by deletion of Tets are already identified using those mice. Using them should facilitate to clarify the importance of Tet enzymes.

5.
There is a possibility that the promotion of regenerative capacity in RGCs by OSK is non-cell autonomous. Indeed previous studies have demonstrated that senescence is induced by forced expression of the reprogramming factors in vivo, and that those senescent cells enhance the cellular plasticity in neighboring cells in a non-cell autonomous manner through secretory components (Mosterio L et al., Science 2016, Chiche A et al., Cell Stem Cell 2017). Labeling OSK expressing cells by introducing a fluorescent protein together with OSK possibly addresses this problem.

Minor concerns:
In Fig. 1b, the authors show that mice infected with OSK AAV have a survival rate comparable to wildtype mice. This is certainly an interesting observation, but this does not have any relevance to the main idea of the paper, or the relevance is not discussed. In addition, the following experiments expressing OSK are performed not in the whole body but in RGCs or in human cells. Therefore, this should go to a panel of Extended Figs.
It seems that in some experiments decent controls are not prepared and/or not used for statistical comparisons. The followings are the examples: In Fig. 2e, ORGs expressing OSK before injury should be included. A statistical test should be done in the same "Injury" group. In Fig.2h, DNAm Age of cells treated with VCS without OSK should be included in the analysis to demonstrate the decrease in DNAm Age is not due to the VCS treatment. Also, OSK expressing cells without VCS treatment should also be analyzed. In Fig. 4i, the statistical comparison should be done between the sh-Scr sample and the sh-Tet1/2 samples under the same condition, e.g. +OSK. Plus, +OSK without shRNAs should be included, as in Fig. 4g and 4h. Otherwise the authors' conclusion that "Four weeks of OSK AAV expression significantly decreased DNA methylation age" is not evidently supported by Fig. 4i.
The authors suggest Tet dependent DNA demethylation have a role in the reprogramming process, but it is still not obvious from the experiments that Tets do not have a role in axon extension/regeneration itself. Seemingly only the comparison between the -OSK sh-Scr and -OSK sh-Tet2 in Fig 4i may address this point, which has no statistically significant difference. Can the authors demonstrate that Tet downregulation have an impact on the reprogramming process?
In Fig. 2i and 2j, neurite area does not appear to be a right metric to assess axon extension capacity and amplifies differences to possibly lead to failure in evaluation (what if a neuron extends only a couple of very long neurite). Length of extended axons or some statistics of length such as mean or madian seem to more accurately reflect the capacity.

Referee #4 (Remarks to the Author):

Comments to the Author

In this study, the authors showed that sustained expression of Oct4, Sox2, and Klf4 improve retinal ganglion cell survival and axon outgrowth in an acute nerve damage and glaucoma model in young and old mice. The effect sizes are sometimes high sometimes less pronounced (see points below). The effect was found to be Tet-dependent implying that DNA methylation plays a role. The strength of the paper is the apparently increased axon growth in various physical damage models of the retina or optic nerve. The weaknesses of the paper are (i) the somewhat unsatisfactory mechanism of what the 3 TFs actually do in retinal ganglion cells (if the mode of action is at all cell-autonomous) (ii) in particular in light of issues of novelty given previous reports that transient iPS cell reprogramming reverses aging-related phenotypes (Ocampo 2016). Moreover, from a practical point of view, I don't think that clinicians that read the paper would be now eager to inject 3 oncogenes into the retina of glaucoma patients. Perhaps if the mechanism is better clarified there might be a more tractable therapeutic angle. In summary, the paper describes an interesting phenomenon that is not well understood and has no direct therapeutic implication. Finally, the writing of the manuscript can be certainly improved, by omitting some far-fetched comparisons in the introduction and better describing the experimental details (see below).

Specific Points:

• Authors should show the data about the risk of tumor formation induced by AAV-OSK expression.

• Knockdown of Tet 1/2 blocked the effects of OSK. Authors should also show the effect of knockdown of Tet 1/2 in axon density (Fig 3d), and RNA-seq pattern (Fig 4e, f).

• If the mechanism is indeed simple DNA methylation, activation of Tet1/2 alone should promote axon regeneratoin and restore vision in these eye disease models.

• The proposed model of the molecular action is underdeveloped. It is implied that the aging methylation marks play functional roles, but the correlations are quite weak and Tet knockdown should affect all DNA methylation, not just the aging-characteristic methylations.

• Fig. 1c: I don't see the controls: without infection, with infection but without dox, with control infection (tTA only e.g.).

• I would be curious to know how OSK were linked together and whether the 3 proteins are expressed at same ratios.

• Are the 3 TFs acting on retinal ganglion cell directly or are they causing other cells to change and secondarily influence axon outgrowth?

• How long is the expression of the 3TFs needed?

• The text could be clarified and better aligned with the figures. E.g. In Fig1d the timing does not match what is described in the text. The CTB treatment shown in that Figure is not described in the text when it is first referred to. These things make it confusing and hard to read.
• It further needs to be explained why the 3 factors delivered separately do not work, but the polycistronic vector does work. I don't see a quantification and statistical analysis in Suppl Fig. 3 that would explain the lack of an effect. At least one would expect to see a partial effect with the separate viruses.

• Another discrepancy is why the number of axons is ~5x higher but the number of ganglion cells only ~50% higher in OSK compared to the average of the remaining conditions. The # of ganglion cells and # of axons should be similar, if anything the # of axons should be lower because you might assume that not all cells extend axons. How much retrograde degeneration is happening? Is the effect thus mostly because of increased axon outgrowth or due to increased cell survival?

• Confusing is also the timing of transgene expression. Fig.1d shows the transgenes were expressed first and then the lesion was induced. In Fig.2 the authors make the statement that transgene activation only after the lesion was helpful. Please clarify.

• Please explain Fig. 1e: All 4 samples are from 1-month old animals? Does this mean that GFP infection dramatically increases the aging methylation signature? If this is true that OSK effects may have to be interpreted differently.

• The neuron outgrowth data in Fig 2j look weak. All neurons shown look very sick. Where are the positive controls? How do we know the culture conditions are permissive for healthy neuronal survival? Primary or human stem cell derived neurons look very different than what is shown. The rescue may seem large compared to the shown baseline but it is far from how normal neurons should look like. Furthermore, it would be more accurate to measure the actual neurite lenghts and branchpoints rather than the area of neurites.

• Use of human immortalized cell lines is not the state of the art anymore. Human ES or iPS cells provide a much better way to obtain human neurons.

Author Rebuttals to Initial Comments:

Referee responses:

We were pleased to see that the referees found our study to be “an exciting paper” that is “well done and the results of high quality,” with findings “of huge importance,” “striking,” “new and interesting,” “very interesting,” “fundamental to neuroscience and medicine,” and of “great potential” for a “new way to counteract age-related diseases and natural aging.”

In response to the referee’s suggestions, we have performed 20 new sets of experiments, added 52 new figure panels, 9 of which were animal experiments, including 3 new transgenic lines and 2 year-long safety studies that we preemptively started last year. We are excited about how much better the paper is now thanks to the referees’ truly excellent suggestions and hope they find our manuscript suitable for publication.

Referee’s comments are in blue italics below and our responses are in black.

Referee #1:

This is very interesting and timely paper.

Why and how the young nervous system can regenerate better than the more mature, aged on can, if fundamental to neuroscience and medicine. I think very highly of the groups involved in this paper- they are true experts in their respective fields. There are some serious issues with the in vivo phenotypes and some of the wording in the text, however. These need resolution. I am not fan of cryptic reviews so I will make the points that I feel need new data, analysis and/or text changes as clear as possible. Again, a very interesting paper with great potential, provided the revisions are made.

Major issues:

1. In the introduction the authors state:

This statement is false: more than a dozen papers now show some degree of recovery of axon growth (many from He lab are among the best in that collection) and several papers from Dr.

He's lab have shown functional recovery, as has work from Benowitz lab (also at Harvard Med), and Lim et al., Nature Neuroscience (2016).

I can only speculate that Dr. He (listed as second to last author) didn't see the final text but this stands out as a major concern and sets the stage for the other 3 major issues:

We appreciate that this sentence was confusing. We have changed the text to clarify precisely how our work is novel compared to other studies. It now reads: *"To date, attempts to reverse damage to the optic nerve have been only moderately successful and no treatments have successfully restored eyesight in the context of late-stage glaucoma or old age.*"

We note that a significant difference between our work and previous work cited by the referee is that, to date, recovery of axon growth following optic nerve crush has only been reported in young 1-2 month-old animals while we now show that our treatment also works robustly in 12 month-old aged animals (see Fig.4b, c).

2. The degree of optic nerve regeneration is modest compared to Park et al. 2008 Science, and work on PTEN/SOCS3 conditional deletions, as well as Lim et al., 2016 and de Lima, PNAS 2012 (Benowitz lab). The fibers shown in the main figures don't extend to the chiasm or beyond (an issue for the functional recovery data; an eye divorced from the targets can't restore function.

We apologize for the confusion. In the crush model, OSK-induced axon regeneration is indeed modest compared to PTEN or PTEN/SOCS3 conditional deletions, but better than others, such as sox11[1] or Lin28[2] overexpression, or Klf4 KO[3]. Importantly, we showed that robust regeneration occurs in 12 m-old animals, in stark contrast to the blockage of long-distance axon regeneration during ageing seen in other manipulations such as PTEN deletion[4]. When OSK is induced for 16 weeks post-injury, we do see nerves reaching the chiasm (see Extended Data Fig.3d), but we do not expect a functional recovery as the recovered axons are still far from the superior colliculus. In our manuscript, we do not claim that OSK can restore eyesight in the optic nerve crush model (we wouldn't expect it), but our data do demonstrate significant OSK-mediated improvement of visual function in models of late-stage glaucoma and normal ageing.

*3. The supplemental data S4 show regrowth into the chiasm but regeneration accessory optic targets (MTN, DTN, NOT) are what is required to restore the functions examined here, both at the level of acuity and at all. 3 papers have shown that those targets and those targets only are the ones that mediate OKR/OKN in the mouse. They do only require a minimum of axons to drive those functions but they need *Some* axons from retina to get there. De-nervation of those targets and re-innervation of them after the treatments here, are necessary to conclude any degree of true functional recovery. The controls don't resolve this currently.*

We don't claim that OSK can restore eyesight in the crush model. What we show is an ability of ectopic OSK expression to improve visual function in a late stage glaucoma model and in aged wildtype mice.

4.The glaucoma model involves gradual loss of RGCs. There are scores of IOVS and J Neuro papers showing some functional recovery in such models. The text here makes it seem as if the authors reached a key milestone. This is simply not the case. "To our knowledge, REVIVER is

the first treatment to reverse vision loss in a glaucoma model" (No)

We agree with the referee that the work of other glaucoma investigators was not adequately documented in the original manuscript. We are aware that there have been numerous studies using neuroprotective treatments in rodent models of glaucoma (see reviews [5,6]) that target a variety of pathways in RGCs, including anti-inflammatory[7-14], anti-apoptotic[15-17], pro-survival[18-22], and metabolic[23,24] pathways. That said, our work differs from these studies because it is the first "reversal" of late-stage glaucoma that restores visual function. We state in the main text at the beginning of the glaucoma section: "Although some treatments can slow down disease progression, it is currently not possible to restore vision once it has been lost." The studies mentioned be the referee initiate treatment *prior to, or during,* the initial stages of glaucoma when intraocular pressure is increasing, but no axonal damage or decreased visual function has yet occurred. While these experiments demonstrate the ability to prevent neuronal damage, how they apply to glaucoma patients at a more advanced stage of disease is unclear.

Another difference is that our study uses the short-term inducible microbead model of elevated intraocular pressure, whereby intraocular pressure is significantly elevated for 3 weeks and returns to baseline by 4 weeks. At 4 weeks post-microbead injection, a significant loss of RGC function and visual acuity was demonstrated by PERG and OMR, respectively. What makes the work unique is that initiating AAV-OSK reprogramming *after* this pressure-induced damage is able to restore the activity of injured RGCs and visual function.

To make the text clear, we have revised the text to cite additional references and make it clear we are reversing a "late-stage" of glaucoma: "Although other treatments initiated prior to, or during the initial stages of glaucoma can slow the progression via anti-inflammatory[7-14], anti-apoptotic[15-17], pro-survival[18-22], and metabolic[23,24], to our knowledge, REVIVER is the first treatment to reverse vision loss at a late stage of glaucoma."

5.Ironically, while they vastly overstated the novelty of their work (see comments and references above) the authors gave up the chance to highlight what is arguably the most important and novel aspect of their findings- that they can perform the intervention (OSK) after nerve injury and see the greatest effects. Previous works (all those listed above in fact) had to be done prior to the injury... which implies limited therapeutic potential or at least creates a need for novel delivery platforms. The ability to introduce a epigenetic or other manipulations to enhance regeneration of already injured CNS axons at any point across the Lifespan is exciting indeed. I would hope the authors would emphasize this point going forward.

Thank you for the compliment. We now better emphasize the novelty of our work.

Minor:

6. Space is limited but please explain Hutchison-Guilford Syndrome.

We changed the text to highlight the aspects of premature ageing and progeria. The sentence now reads, "Ectopic, transgene-mediated expression of these four genes *in vivo* alleviates symptoms in a premature ageing mouse model of Hutchinson-Gilford progeria syndrome".

7. Ditto for Shannon Information Theory and aspects of which are relevant for stored copies of genetic info. I'm familiar with this but the mention of it in the context of a short paper (several times) without much meat or context, makes it confusing for the non-expert.

For clarity, we have deleted mention of Shannon's Information Theory.

8. The authors should mention the Kennard Principle: the "negative linear relationship between age of a brain lesion and the outcome expectancy: in other words, that the earlier in life a brain

lesion occurs, the more likely it is for some compensation mechanism to reverse at least some of the lesion's bad effects".

We appreciate your keen insight and now mention the Kennard Principle.

9. The display of the retina with RGCs at the top of the image will make Ophthalmologists pleased and neuroscientists confused, despite the location of RBPMs staining. This is not critical to my assessment at all, but consider flipping these to match the convention in 99% of the papers.

Good point. We have now flipped all of the retinal images.

Again, an exciting paper. Epigenomic control of regeneration capacity is fundamentally interesting. There are several technical gems here too: the use of the TET system, the AAVs themselves and the gymnastics the authors went through to get large sequences into them and expressed in vivo. These are not lost on me. The authors are to be commended for the work.

Thank you. We are grateful you appreciate the difficulty of these experiments and the work that went into them.

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Additional points:

10. There appears to be a dramatic decline in the number of axons regenerated between the Tet-On and Tet-Off system, i.e. Fig 1e and Fig. 2c. Please explain the discrepancies and how the system used might contribute to this difference.

The reason for these discrepancies is most likely the fact that the tTA (Tet-Off system) is always on, thereby activating the TRE promoter, whereas the rtTA system (Tet-On system) relies on the presence of Dox in the tissues to activate. The latter depends on when and how much the mouse is drinking.

11. The decline in DNA methylation age seen with human neuronal cultures needs to be controlled better to really establish the similarity in effects between mouse and human. While the cultures treated with OSK are much higher in age before VCS treatment (fig. 2h), the control cultures in extended Fig .8e are comparatively younger before VCS.
Further, in the control cultures, VCS treatment only seems to have a marginal effect on day 1, with some decline in age at day 9. With this in mind, please tone down the conclusions about how conserved the effects of OSK are.

We thank the referee for reading our work so carefully. The conditions used in these two experiments were not identical and therefore are not directly comparable. As suggested, we have toned down our conclusion and we have converted the Y-axis to ΔDNAm Age so the graphs are no longer confusing. The revised conclusion now reads: "~~Paralleling the RGCs,~~ DNA methylation age was significantly increased after VCS-induced damage of human neurons"

12. The authors have stated that the increase in visual acuity restores about half of vision loss...This is an exaggerated statement as they have only shown one behavior that is partially recovered.

We have improved our wording to better reflect the data, i.e. "restoring about half of the visual

acuity loss in the OMR assay."

Further, in Fig 3f and h, they have not indicated if the recovery in visual acuity is significant between the saline and +OSK groups.

The saline intact group is statistically better than all bead-treated glaucomatous groups including Beads (+OSK) group in both Fig 3f and h. We think this statistic is not necessary to show because we are not claiming a full recovery by OSK. We have put all data in the source table so readers can analyze the raw data themselves.

13. In the glaucoma model, the authors state that 'the axon regeneration is slightly less than young mice two weeks after injury (fig. 4b)'. Presumably they are talking about the Tet-Off group in Fig 1 and not the Tet-On group? Please clarify.

You are correct. We are talking about the Tet-Off group. We have changed the text to be clearer: "OSK treatment using the Tet-Off system ".

Referee #2:

In this manuscript, the authors examined the impact of Oct4/Sox/Klf4 (OSK) co-expression on retinal ganglion cell (RGC) axon regeneration and function. They provided evidence that sustained expression of OSK promote axon regeneration in the crush and glaucoma models as well as promote visal function in aged animals without axon regeneration. Such manipulates leads to changes in DNA methylation of a few age-related marks, which as well as axon regeneration requires functions of Tet1 and Tet2. The experiments appear to be well done and the results are of high quality.

We appreciate the very helpful suggestions and the kind words about our work being "well done," "high quality," "new and interesting."

The author started in the introduction by discussing reversal of aging. The authors then provided evidence showing that only when OSK are sustained expressed after injury axon regeneration can be induced, therefore this approach does not reprogram the RGCs to a youthful state as in development (which can regenerate axons), but instead promotes axon regeneration directly.

We agree that this new concept of age reversal needs further clarification. Reversal of ageing can mean two different things. In the case of development, increased recovery from nerve crush could be due to a more youthful DNA methylation age that exists before the damage or it could be due to them having a lower baseline age that makes the cells more tolerant to the advancement of DNAm age post-damage. The latter situation is what we see in our experiments with nerve crush and vincristine damage. OSK counters the advancement of the DNAm clock and actually reverses it, even in the face of damage (see Fig.2h). We agree that it is difficult to prove that age reversal is the reason for the axon growth in the nerve crush experiment because the advancement is happening concurrently with reversal. That is why we then tested DNAm reversal in the neurons of old mice, where there is no concurrent rapid advancement of the DNAm clock. In these experiments, we were able to show that OSK indeed reverses not just the DNAm age, but also restores a more youthful gene expression pattern and the function of younger neurons. We have revised the text to clarify our interpretation of these results and to ensure that we are not making any claims the data do not support.

A number of studies have already shown various ways to promote axon regeneration of RGCs and the concept of reprogramming to promote axon regeneration is not particular novel (e.g.

PMID: 19815778; 28641110).

This will be the first report to show that these three reprogramming factors (Oct4, Sox2, Klf4) can promote axon regeneration. The two references above don't show reprogramming promotes axon regeneration.

Reference PMID:19815778 shows the opposite to what the referee is suggesting. This paper shows that RGCs *lacking* KLF4 have increased axon growth, indicating that, in this context, KLF4 functions as a repressor of axon regeneration. Because we were aware of the KLF4 KO literature[3], we tested whether leaving out KLF4 from OSK improves regeneration beyond what we see with the three OSK factors. It does not. We show that KLF4 needs to be provided along with Oct4 and Sox2 to promote axon regeneration (Fig. 1d fifth dark red line). Overexpression of Klf4 alone is not sufficient (Fig. 1d fourth dark green line). Together these data challenge the statement that KLF4 is a *repressor* of axon regeneration and actually demonstrate that Klf4 can work with other factors to *promote* axon regeneration.

Reference PMID: 28641110 Fig 1b shows Sox2 overexpression alone does *not* promote axon growth. This paper focused on Sox11, which is not considered a reprogramming factor.

DNA demethylation pathway and tet proteins have already been shown to be required for effective PNS and CNS axon regeneration in mice. Therefore, the conceptual advance of the current study is limited.

Tet1 has been shown to facilitate PTEN-mediated axon growth but the mechanism of PTEN regeneration was thought to act by inducing metabolic changes via mTOR activation, which we show is not the case for OSK-mediated regeneration. Our work shows that reprogramming factors can induce neuronal regeneration and restore the function of damaged and old neurons, even restoring eyesight in a late-stage glaucoma model by resetting the age of cells and their gene expression patterns. The fact that other studies have found the Tet genes to be relevant to neuronal regeneration[25] merely speaks to us having discovered a process with broad implications for both the PNS and CNS. It is interesting to speculate that the PTEN KO results are relevant to the process we describe but knocking out a tumor suppressor in the eye is not likely to lead to safe therapies whereas ours seems remarkably safe both in the retina (Extended Data Fig. 2 c-i) and systemically (Extended Data Fig. 1 j-n).

It is worth noting that after injury to the PNS, adult neurons can naturally switch from a dormant state with low regenerative capacity to one with robust axon regeneration (in contrast to the CNS). Therefore, the observation that Tet3 is required for PNS regeneration in adult neurons suggests the exciting possibility that active DNA demethylation by OSK within the CNS may work by mimicking the PNS switch, a possibility we plan to discuss in an upcoming review.

On the other hand, the application of their system in the Glaucoma and aging models is new and interesting.

Thank you.

Major concerns

1. The authors need to perform gene expression analysis of RGCs at different ages and upon OSK expression with or without injury. Does OSK expression leads to expression of regeneration associated genes in RGCs after injury? How about the DNA methylation status of these regeneration associated genes? The authors have the RRBS datasets in hand.

Thank you for this suggestion. We have re-analyzed our RNAseq data sets to see if regeneration-associated genes are altered significantly (Supplement Table 1, see below). In the aged mice treated with OSK, there was a significant change in many regeneration-associated genes. Of these, Stat3 was most significant (Q = 5.84 E-13). Hyperactivation of Stat3 has been widely reported to promote axon regeneration[26-28]. Based on qPCR, OSK upregulates Stat3 7-fold at the mRNA level (Fig. A below) and shRNA knockdown of Tet1 or Tet2 mRNAs partially blocked STAT3 upregulation (Extended Data Figure 6g, see below). To confirm this result, we examined STAT3 protein levels by staining whole mounts of retinas with or without injury and saw that the pSTAT3 signal 3-days post-crush is greatly reduced compared to pre-crush neurons (Extended Data Figure 5a). Importantly, OSK-infected neurons maintain a strong pSTAT3 signal even after crush, suggesting pStat3 is an important pro-regeneration protein downstream of OSK treatment. We have added these exciting new findings to the manuscript. Thank you.

Based on the referee's advice, we also analyzed the RRBS data and see a trend towards demethylation of the STAT3 promoter in the OSK-infected neurons post-crush (Fig. B below). Because of the low coverage of the RRBS at non-repetitive genome regions, we only see a trend, and therefore have not included this data in the revised manuscript, but have included the figure below for the referee's information.

Extended Data Figure 5a

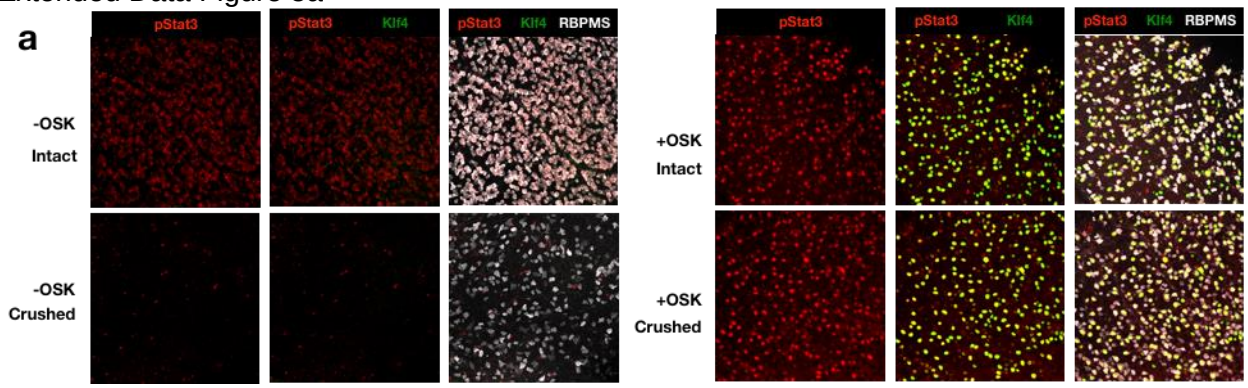
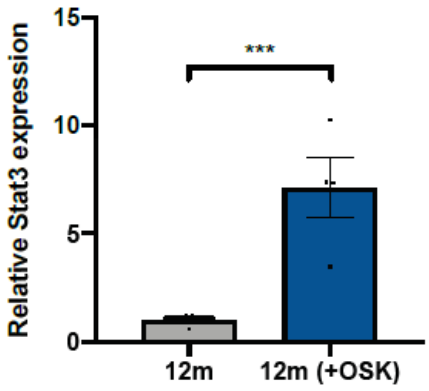


Fig. A (data not included in paper):



Extended Data Figure 6g:

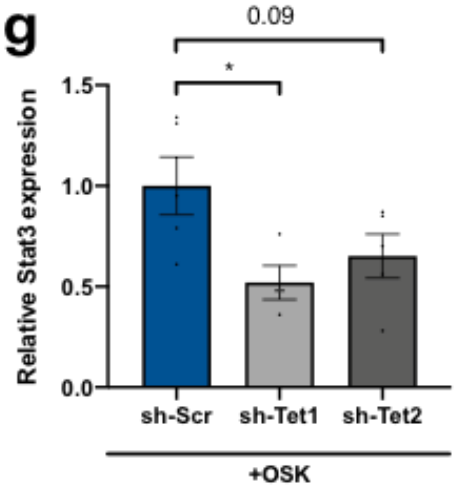
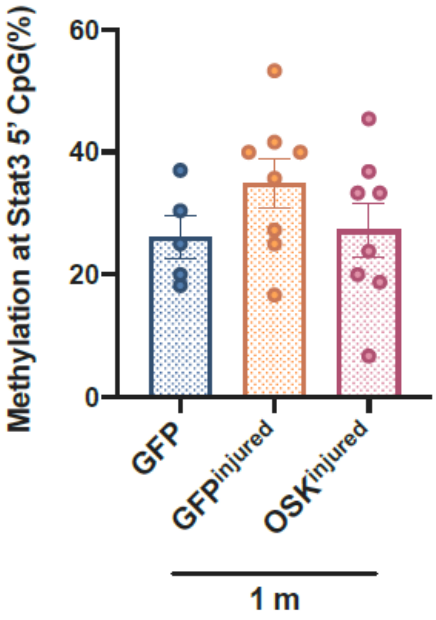


Fig. B (data not included in paper):



2. Given only 70% overlap of shRNA-Tet1 or Tet2 expression with OSK vectors, you would expect still see significant number of regenerating RGC axons. But the results showed no axons longer than 500 µm (Fig. 2f).

This is an astute observation. We do see that some regeneration in the shTet-infected neurons, but the length of the regenerating axons is less than that the sh-Scr control. This is not surprising, howver, given that THY1-YFP (RGC-specific expression of YFP) is known to have shight toxicity in the context of axon regeneration. In our lab (Zhigang He's), for example, homozygous expression of THY-YFP in our YFP17 transgenic line inhibits axon regeneration in the PTEN KO mice and this toxic effect is significantly reduced with heterozygous expression of THY-YFP. Consistent with this, sh-Scr YFP-expressing mice have reduced axon regeneration compared to mice without sh-Scr YFP.

For numerous reasons axon regeneration might not be directly proportional to infection overlap, including increased cell death in the shTet1 or shTet2 mice, which could inhibit the regeneration of adjacent axons and the fact tha there are 46 different RGC cell types[29] and the likelihood that some of the neurons that receive the shRNA are not RGC subtypes that can regenerate.

3. The experiments with SH-SY5Y cell lines (a tumor cell line, developmental immature neurons) is not very relevant and should be removed (Fig. 2g-j).

We considered a few options: human ESCs, iPSCs, or a human neuronal cell line. Unfortunately, ES and iPSC cells are not feasible for age reversal studies because passage through the pluripotent state resets age and phenotypes[30], and as such their epigenetic age is zero (ref. [31] Fig.5a-c). Because we are studying ageing, we selected a cell line that starts out with an older baseline and now explain this choice in the revised manuscript.

4. In the glaucoma model, the authors need to show regeneration axons, instead of axonal densities. What's the infection rate for OSK expression in this model? The recovery is almost complete (Fig. 3d). Is the effect completely cell autonomous?

The infection rate in the glaucoma experiments was ~30% (Extended Fig.9c). In the glaucoma model, we know the RGC number does not increase (Extended Fig.9d). Therefore, the recovery of axon number in the late-stage glaucoma model is most likely due to *axon sprouting* rather than axon growth. In the glaucoma model, there are many overlapping axons in longitudinal sections (~500K), making it technically impossible for us to count axons in glaucoma by CTB but sprouting can be clearly seen in the crush model where there are far fewer axons and these changes are visible (see Fig.4c).

We have added multiple lines of evidence in the crush model indicating that the OSK effect is largely cell-autonomous and RGC-specific (also see the response to your point 8, to Referee 3 point 8, and to Referee 4 point 7).

5. The authors should also perform optomotor response behavior test in the axon crush model for young and old animals.

One of our labs (Zhigang He's) has one study that restores visual function in a crush injury model with PTEN/SOCS3 deletion, but even then, the crush site was adjacent to the brain not the eye, and that treatment needed to be combined with a potassium channel blocker[32] (Bei, et al., 2016 *Cell*). In our experiments, where the crush was adjacent to the eye, the number of axons connecting back to the visual cortex was low, even after 4 months of OSK expression. For these reasons, the regeneration would not be expected to register in OMR assays. A future experiment would be to repeat these experiments in the presence of a potassium channel blocker to try to improve axon growth, but such experiments are well beyond the scope of this study.

6. The RNA-seq analysis of 5 months and 12 months old animals are interesting. Does the

recovery of gene expression to levels of 5 months old animals by OSK expression require Tet1 or tet2?

Based on the referee's suggestion, we attempted this RNA-seq experiment, but after considerable work found it was not technically feasible. The number of RGC that can be sorted from a mouse retina is already low (<10K) and is compounded by the fact that we needed to use FACS to isolate the YFP-positive shRNA-containing cells, leaving us with insufficient material to do genome-wide bulk RNA-seq analyses (see the table below).

Institute: Harvard Medical School
Quotation Number: 30-220583634

Sample/Name	GENEWIZ ID	Sample Type	Sample Vol. (ul)	RNA Qubit			
				Dilution Factor	Nucleic Acid Conc. (ng/ul)	Actual Nucleic Acid Conc. (ng/ul)	Total Amount (ng)
12m-shcntl-1	DV01-12m-shcntl-1	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shcntl-2	DV02-12m-shcntl-2	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shcntl-3	DV03-12m-shcntl-3	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shcntl-4	DV04-12m-shcntl-4	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shcntl-5	DV05-12m-shcntl-5	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shcntl-6	DV06-12m-shcntl-6	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet1-1	DV07-12m-shTet1-1	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet1-2	DV08-12m-shTet1-2	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet1-3	DV09-12m-shTet1-3	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet1-4	DV10-12m-shTet1-4	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet1-5	DV11-12m-shTet1-5	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-1	DV12-12m-shTet2-1	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-2	DV13-12m-shTet2-2	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-3	DV14-12m-shTet2-3	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-4	DV15-12m-shTet2-4	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-5	DV16-12m-shTet2-5	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW
12m-shTet2-6	DV17-12m-shTet2-6	total RNA	18	1	TOO LOW	TOO LOW	TOO LOW

We were able to generate cDNA from these minute quantities of RNA using the SMART-Seq v4 Ultra Low Input Kit for quantitative PCR. Using this material, we were able to validate the knockdown of Tet1 and Tet2. We also examined the existing RNA-seq data from young versus old RGCs to gain insight into which genes downstream of OSK might be responsible for the recovery of vision. By cross-referencing our data sets with lists of genes that play a role in retinal ganglion cells axon guidance (http://amigo.geneontology.org/amigo/term/GO:0090259), or involved in neural projection development (http://amigo.geneontology.org/amigo/term/GO:0010977), we narrowed the list down to three genes, namely Efemp1, Ngfr and Trpv4 (Extended Data Fig.10e). Of these three, only Efemp1 was abundant enough to be measured by qPCR.

Efemp1 is very interesting and relevant gene. For example, Efemp transcription increases during ageing, is a known negative regulator of neuronal projection development, missense variant of Efemp1 is found in human open angle glaucoma cases[33], and the accumulation of Efemp1 protein in RPE cells is linked to drusen formation in age-related macular degeneration (AMD) and Malattia Leventinese (ML) disease, a disease similar to AMD[34]. Efemp1 has also been identified as a novel amyloidogenic protein in humans, as it frequently forms amyloid deposits in systemic venous walls of elderly people[35].

Using qPCR, we validated the RNA-seq results showing that Efemp1 expression is two-fold higher in old vs young RGCs, and OSK treatment of aged RGCs reversed this age-dependent increase. In our newly prepared cDNA samples, we find that Tet1 and Tet2 are required for the restoration of Efemp1 mRNA levels (see Extended Data Fig.10f).

All was not successful. We attempted to analyze the methylation patterns of the Efemp

promoter region but unfortunately the sequencing depth at this locus in the RRBS was not high enough across all samples to compare DNA methylation levels and our attempts to stain Efemp protein in aged retinas was unsuccessful due to limitations of the available antibody.

The other two genes unrelated to retinal function, i.e. Map6, Sgpp2, which were significantly downregulated by OSK, were unaffected by Tet1 or Tet2 knockdown, suggesting they may not be relevant to the restoration of vision.

7. How about gene expression in response to injury at two different stages with or without OSK expression?

This an interesting question given that we observe OSK-induced axon regeneration being slower but not prevented in aged mice. But it is a question that will be harder to interpret without much deeper analysis. Considering that axon regeneration in response to CNS injury in ageing involves extrinsic paracrine influences at the injury site from other cell types such as astroglia, microglia, and macrophages[4], we plan on investigating this as a follow-up set of experiments in the next study because it is beyond the scope of this one.

Other comments

8. Are all regenerated axons with OSK expression?

When looking at whole flat-mount retinas, there is a high concordance between OSK expression and cell survival. With a starting infection rate of 40% without injury and 70% at two weeks post-injury (new Extended Fig.4g), and 90% at five weeks (new Extended Fig.9h), the survival effect is mostly cell-autonomous.

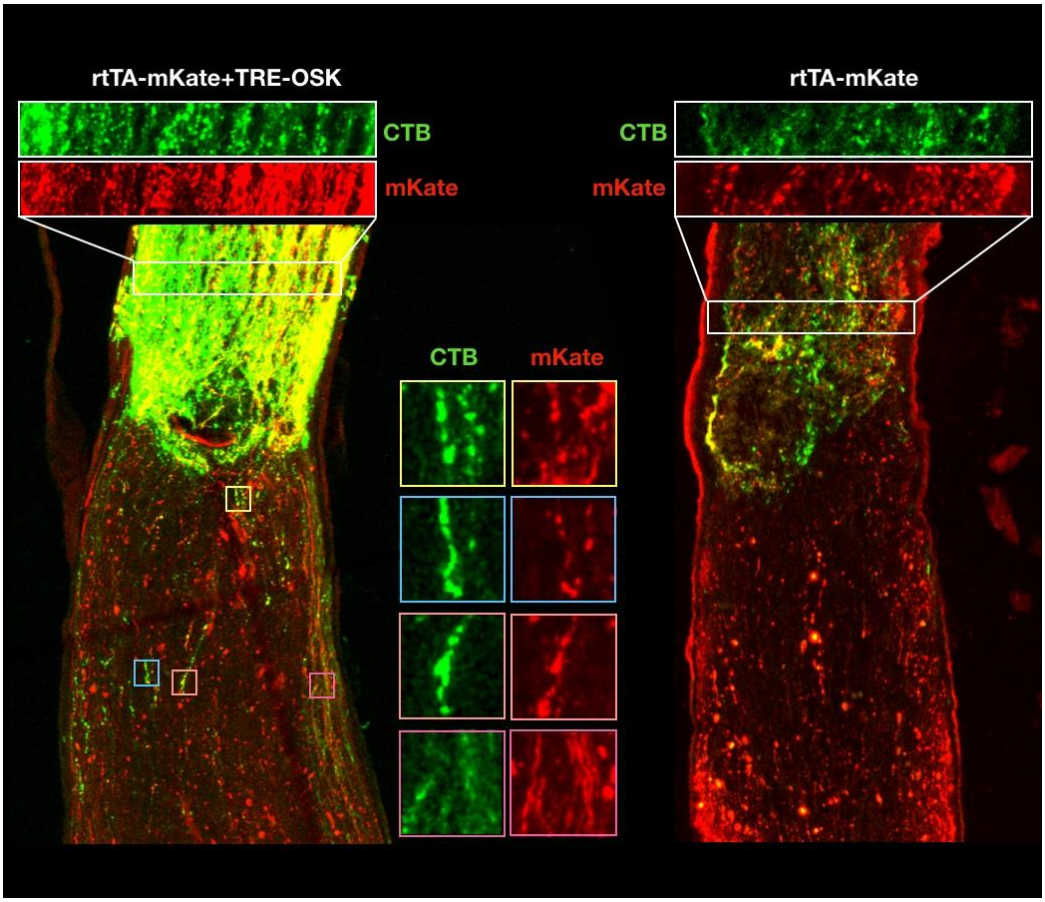
Whether or not the axon growth is also cell-autonomous is more difficult to answer than it might seem. The issue is that Oct4, Sox2, and Klf4 are nuclear proteins and do not travel down the axon. The ideal solution is to link a reporter protein to OSK with an IRES or 2A peptide, as referee 3 suggested. However, the AAV can only package <4.7kb that is already occupied by OSK and other necessary components (i.e. the promoter and SV40). We cannot not use lentiviruses as they don't transduce RGCs.

To get around this problem, we linked mKate to the rtTA gene (rtTA-2A-mKate) in the other AAV of the dual AAV Tet-On system and co-injected it with TRE-OSK AAV, then treated with Dox after crush. In these experiments, there were two possible outcomes. Either,

1. Almost all of the regenerated axons were mKate positive. This would be consistent with OSK-mediated regeneration being ***cell-autonomous*****.**

2. A minority of regenerated axons were co-stained with mKate and CTB tracer, and their percentage was lower than the mKate AAV infection rate of RGCs (~50%), which would be consistent with OSK-mediated regeneration being ***non*** cell***-autonomous.***

What we observe is consistent with result #1 (shown below). Though the regeneration is mild due to the weaker efficacy of rtTA compared to tTA (see Referee 1 point 10), in the OSK group (left), with CTB in green and mKate in red, most surviving axons before the crush site were mKate positive, and those past the crush site had mKate along their axons. mKate wasn't seen along the entire axon because it is a cytoplasmic protein that is known to be transported inefficiently. By contrast, in the control mice, only a minority of axons overlapped with mKate (right). The limited signal of CTB (green) in the right control sample is expected because cell death is prevented in the OSK-treated sample on the left. These data are consistent with OSK-mediated survival and regeneration being cell-autonomous.

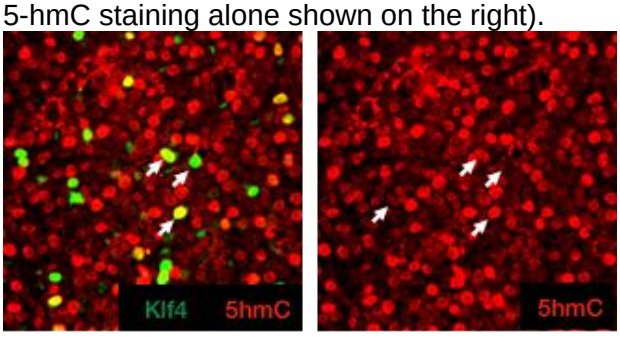


9. How long after injury can expression of OSK still promotes axon regeneration?

This is a tricky question to answer precisely because there are a few variables. Firstly, there is a delay in OSK expression because the mice have to wake up from anesthesia and start drinking Dox-containing water but full induction of OSK takes about 2 days. It is known that the apoptosis of crushed neurons peaks at Day 4[36], so we suspect we are within the window of opportunity and that later time points will be far less effective. We have only tested the effect of inducing OSK within the first 24 hours post-injury, but plan to test longer wait times in future studies and certainly agree this is an interesting line of investigation.

10. Does OSK expression lead to elevation of 5hmC levels in RGCs after injury?

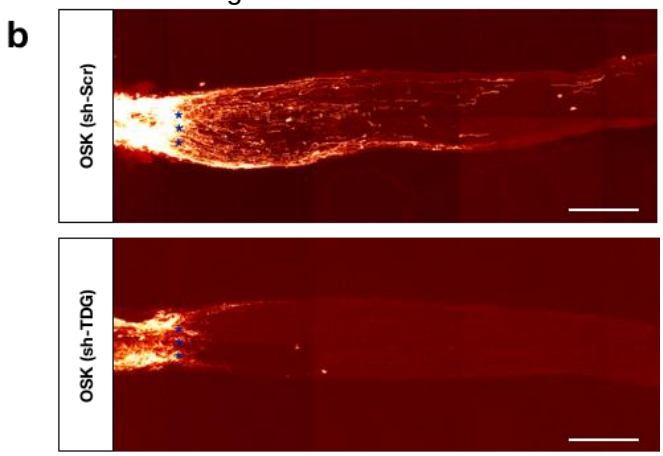
As we showed using RRBS and whole mount staining (Extended Data Fig.5g, h), unlike during iPSC generation, OSK expression in post-mitotic RGCs does not induce global changes in 5mC, so we don't expect a global elevation of 5hmC levels. Prompted by the referee, we stained OSK-infected RGCs for 5hmC levels and didn't observe any clear consistent differences compared to uninfected RGCs (see below, white arrows point at Klf4 infected RGCs on the left, 5-hmC staining alone shown on the right).



11. Tet proteins are known to exhibit functions independent of active DNA demethylation. A confirmation of role of active DNA demethylation requires other manipulations, such as downstream Tdg manipulation (PMID: 28426967).

Excellent point. To address this, we first ruled out the possibility of passive demethylation by performing BrdU labeling of neurons, which showed OSK-infected cells do not divide (new Extended Data Fig.3b). Then, as suggested, we investigated whether the downstream mediator of active demethylation, TDG, a thymine DNA glucosidase, is also required for axon regeneration *in vivo*. We contacted Yi-lan Weng and Hongjun Song who published a paper in 2017 on the role of TDG in peripheral nerve regeneration[25]. They kindly shared their TDG knockdown AAV and corresponding control plasmids. The AAVs were co-injected intravitreally together with +OSK AAV. The expected effect of the TDG knockdown in normal RGCs was seen, i.e. the level of 5hmC was elevated (Extended Data Fig.7a). As shown in Extended Data Fig.7b-d, knockdown of TDG completely abolished the beneficial effects of OSK on both axon regeneration and RGC survival post crush injury. These data strongly indicate that enzyme-dependent active demethylation is required for OSK to regenerate axons (Extended Data Fig.7h). We have added this data to the manuscript and are grateful to the referee for the suggestion.

Extended Data Fig.7b



Referee #3:

The manuscript from Lu and colleagues reports striking finding that in vivo expression of Yamanaka factors confers on RGCs axon regeneration capacity after injury, which is lost after birth. They further show that this axon regeneration leads to vision recovery in glaucoma model mice and in normal aged mice. These findings are of huge importance in that the extent of the axon extension and the vision recovery has been vastly improved compared to other studies as of today. Moreover, through their observation that the DNA methylation level correlates well with the regenerative capacity, they set out to examine the functional roles of Tet1/2 demethylases by introducing shRNAs against Tet1/2. Their results suggest that altered DNA methylation is associated with acquisition of the regenerative capacity by OSK. The current study sheds light on modification of DNA methylation as a new way to counteract age-related diseases and natural aging.

We thank referee 3 for these positive comments.

Major concerns:

1.

As the authors mention in the manuscript, it has been well established that the level of DNA methylation at particular locus is gradually increased along aging and that it works as an aging biomarker, also known as the DNA Methylation Clock. However, what the functional roles of the DNA Methylation Clock in aging are and whether reversing the clock can alleviate functional decline by aging are still unknown and, therefore, a subject many researchers put a ton of effort into.

The authors demonstrate that expressing the reprogramming factors can indeed decrease the DNA methylation level at the rDNA regions (rDNAme clock), which prompts us to assume that rDNAme might have a link to the acquisition of the regenerative capacity. However, given that tons of molecular events are provoked and multiple pathways are activated downstream of the OSK expression, I would have to say the experiments in the manuscript fail to successfully exclude the possibility that the reversal of the rDNA clock and the acquisition of the regenerative capacity are independent events and have no causal relationship.

We agree that there are many events downstream of OSK. One of the most important was to know if *active* demethylation is required for axon survival and regeneration. To determine this, we tested if TDG, the enzyme downstream of the Tets required for active demethylation, was necessary for axon survival and regeneration by OSK, and it was (see Referee 2, point 11 above). The fact that both Tet1/2 and TDG are necessary for OSK to mediate its effects strongly argues that active DNA demethylation is indeed required and that the rDNAm clock is one readout of this activity. The Tet and TDG results, while compelling, do not prove that the clock itself is required. In the manuscript, we are careful to clearly state that alterations in DNA methylation patterns, as indicated (not caused) by the rDNA methylation clock, may participate in regeneration and in ageing.

2. Having said that, the observation that downregulation of Tet1 or Tet2 suppresses the OSK-dependent youthful phenotypes strongly suggests that erasure of DNA methylation is involved in the process of "rejuvenation".
Considering Tet1 and Tet2 control DNA demethylation genome-widely, it is unlikely that alteration in DNA methylation can be seen only in rDNA regions. Therefore, the authors should analyze DNA methylation changes not only in the specific locations, e.g. rDNA loci, but also in an unbiased manner. An analysis of global DNA methylation profiles in a decent set of samples, such as -/+ OSK, -/+ Tet1/2 alone is invaluable, as it will certainly facilitate to understand the molecular mechanisms by which OSK confer regenerative capacity on RGCs.

In response to the referee's valid suggestions, we examined methylation globally using RRBS, including CpG methylation patterns in gene bodies, promoters and CpG islands[37]. From a total of 68 DNA samples, there were no significant changes in global DNA methylation patterns during ageing or OSK treatment (see Extended Data Fig. 5i, shown below). This is consistent with the lack of change in 5-mC signals in whole mount retinas in OSK-expressing RGCs (see Extended Data Fig. 5g). However, when we examined DNA methylation changes at the promoters of Sox2 and Stat3 (see Fig. C below), there was a trend of towards a reduction in DNA methylation in the OSK group, which would be consistent with the increase in their mRNAs after OSK expression. These data are also consistent with some of our main findings: (i) that OSK does not work via a genome-wide, passive demethylation process but active demethylation, and (ii) that OSK expression induces site-specific changes to DNA methylation pattern which includes clock sites.

Extended Data Fig. 5i

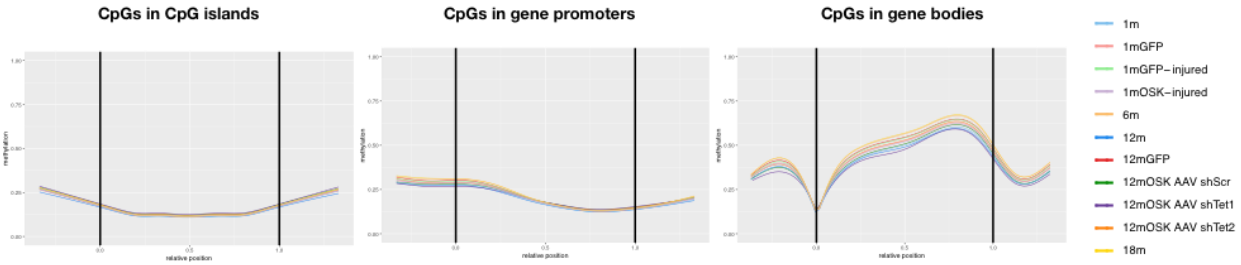
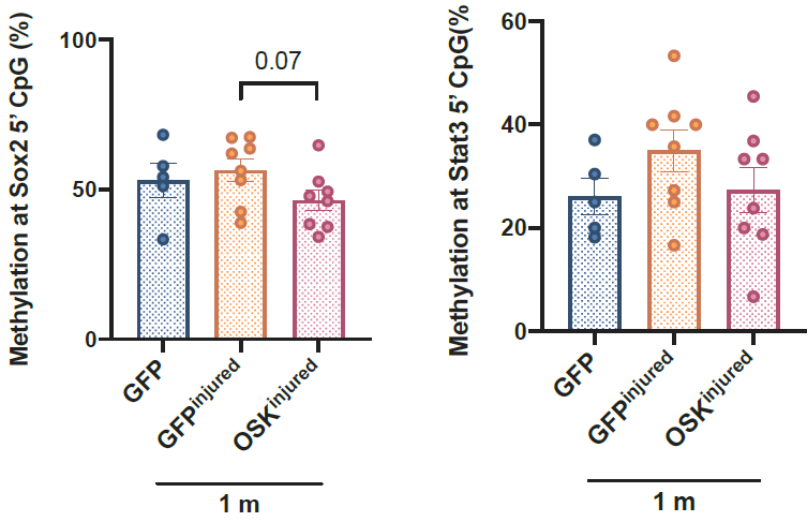
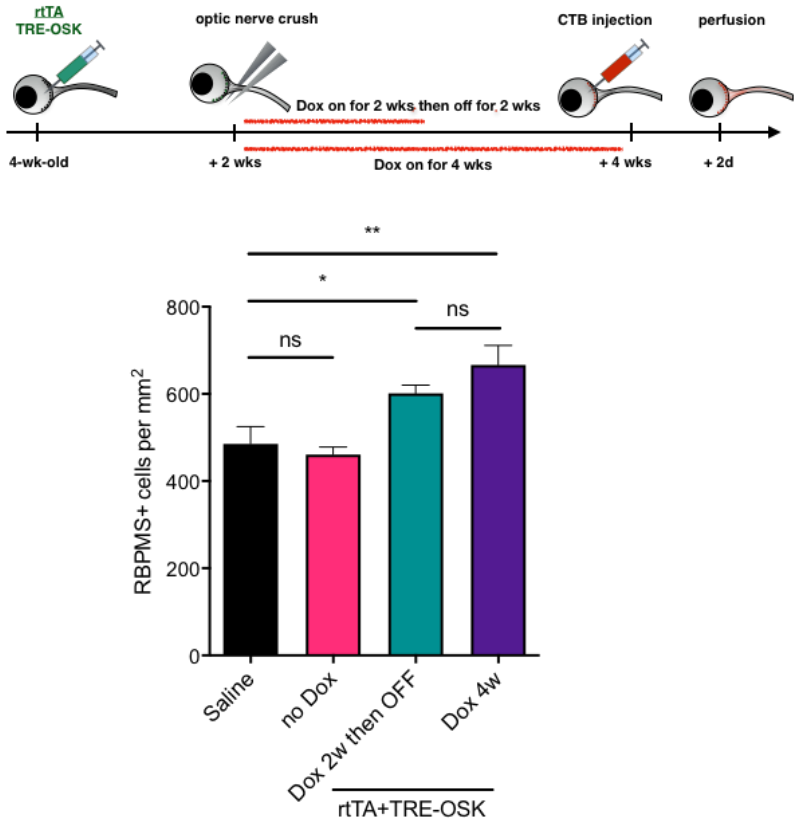


Fig. C (Data not shown in the paper)



3. When such DNA methylation analysis is performed, a sample in which Dox is withdrawn after OSK-mediated regeneration should be included, as OSK expressing cells presumably have molecular profiles far apart from normal RGCs with OSK being turned on.

This is an excellent suggestion. In the optic nerve crush model, we have now tested the effects of continuous OSK expression for 4 weeks following crush injury (show in the original manuscript) with the effect of expressing OSK for only 2 weeks after crush injury and then withdrawing Dox and turning off OSK expression. The results below demonstrate that if we turn on OSK for 2 weeks and then turn it off for 2 weeks, RGC survival is maintained and is not significantly different from mice in which OSK expression was continuous for 4 weeks. These data will form the basis of a follow-up study, and therefore are not included in the revised manuscript.



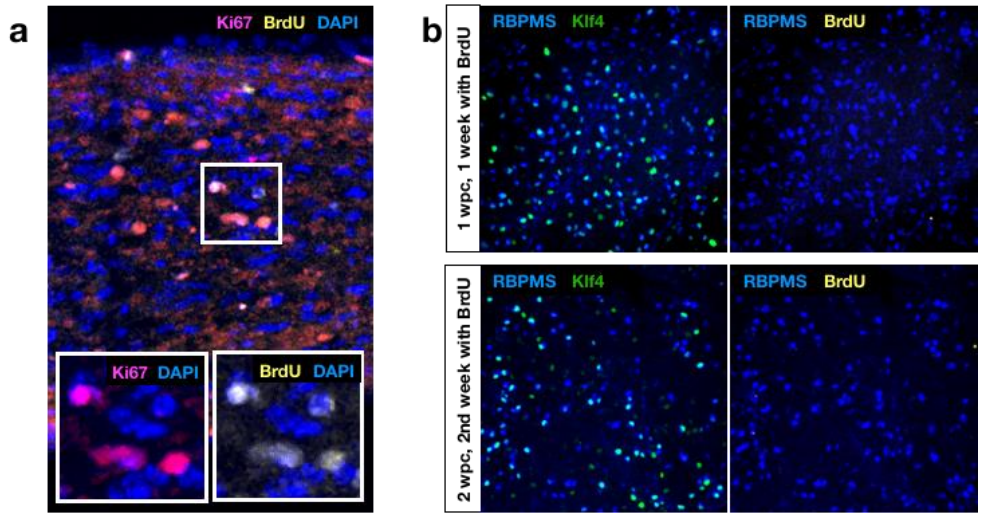
4.DNA demethylation occurs through an active pathway and a passive pathway, which are through cell division and by TET DNA demethylases, respectively.

As such, it is critical in the current study to demonstrate that RGCs do not proliferate by the OSK introduction. The authors indeed show no upregulation in Ki67. However, this alone does not convincingly support the statement. The authors should conduct additional assays, such as BrdU incorporation in a quantitative way, to make the statement more convincing. In Fig. 1f, the authors show the numbers of RGCs per mm2 16 hours after the nerve crush as survival rates. The authors should also examine time course changes in the numbers of RGCs after the crush to confirm the lack of cell proliferation. This would give clearer interpretation since the positive control for the immunostaining in Extended Fig. 4a is not the best one, which is HEK293 cell line.

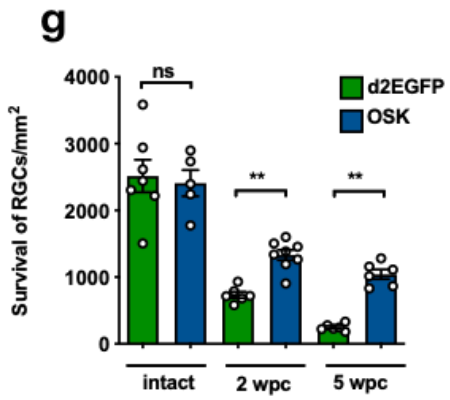
This is an excellent suggestion. In response, we performed daily intra-peritoneal BrdU injections during the first or the second week post-crush injury. Proliferating cells such as glial cells in optic nerve from the BrdU injected mice served as positive controls for proliferating cells. We also co-localized BrdU staining with Ki67 to confirm that we were detecting proliferation (Extended data Fig.3a). As shown below and in Extended data 3b, no BrdU expression was detected in the RGCs, including OSK-expressing RGCs. This fits with our previous observation that in the context of glaucoma and ageing, OSK expression does not increase RGC number (Extended data Fig.9d and 9k).

As the referee also suggested, we performed a time-course of RGC survival in the optic nerve crush model (Extended data Fig.9g). After injury, even with OSK treatment, we observed reduced RGC survival over time. While the rate of RGC loss was slower in the OSK-treated group, when compared to the GFP group, there was no increase in the number of RGCs, further indicating there is no proliferation of RGCs in the OSK group.

Extended Fig. 3a and b



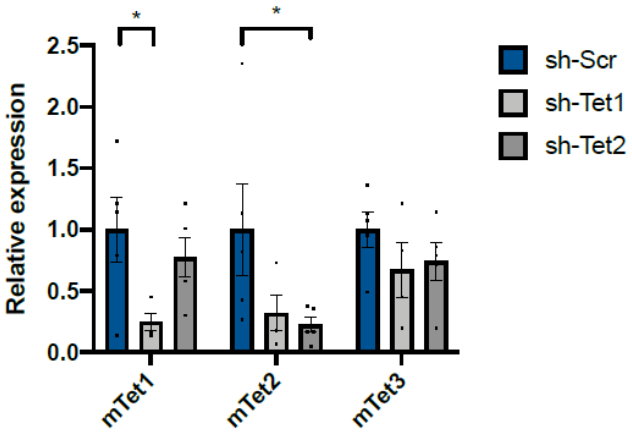
Extended Fig. 9g



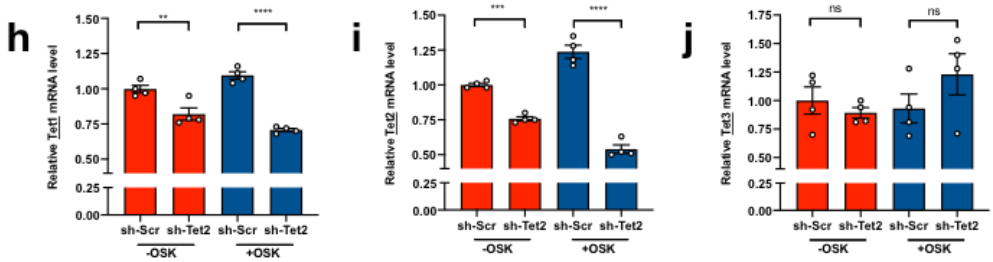
5. Tet1 and Tet2 are known to be, at least in large part, functionally redundant. Thus downregulating only either of the two are more likely to have no or little effect on DNA methylation. This point should be addressed, or at least discussed in the manuscript.
On that note, the expression levels of Tet1 and Tet2 are not examined in the experiments using shRNAs against them. The authors need to add the data to show how much the suppression efficiency is. This will also provide the information how specific these shRNAs are in the RGC context.

Great suggestion. To address this, we extracted RNA from RGCs and measured mRNA levels of Tet1 and Tet2 using qPCR. Similar to previous publications using the same shRNAs[25,38,39], Tet1 knockdown was 75% and Tet2 knockdown was 78% (Extended Data Fig.6d). Interestingly, knockdown of Tet1 also reduced Tet2 mRNA levels, consistent with previously observed co-regulation (Etchegaray *et al*., *Nature Cell Biology* Fig.4e[40]). In human SH-SY5Y cells, knockdown of Tet2 also reduced mRNA levels of Tet1 but not Tet3, further corroborating this result (Extended Data Fig.8h-j).

Extended Fig. 6d



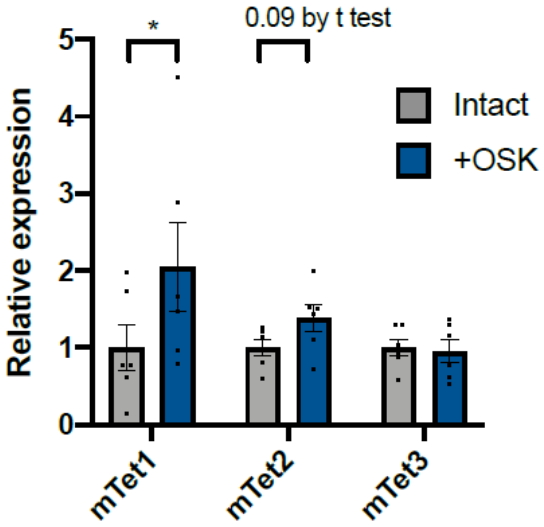
Extended Data Fig.8h-j



6. In addition, the expression levels of Tets in RGCs and those in RGCs expressing OSK should be examined.

We have now measured mRNA levels of Tet1, 2 and 3 in RGCs after OSK induction for one month. OSK robustly upregulated Tet1, slightly upregulated Tet2, and did not affect Tet3 mRNA levels (Extended Data Fig.6a shown below), matching what has been previously reported for fibroblast reprogramming *in vitro*[41,42]. There was insufficient material to measure protein levels.

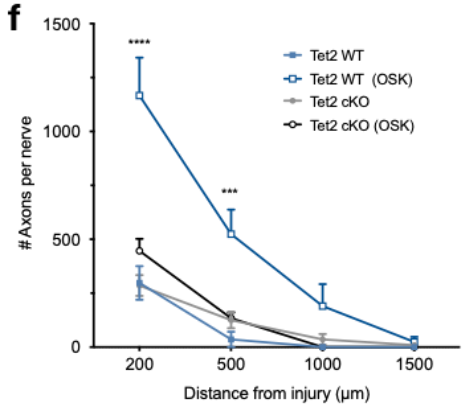
Extended Data Fig. 6a



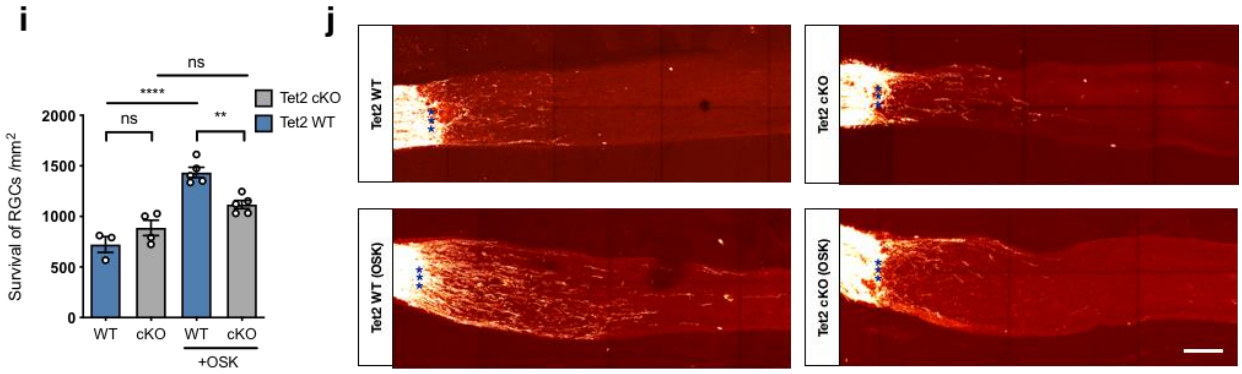
7. Tet1 KO mice and Tet2 KO mice were previously generated (Dawlaty MM et al., Cell Stem Cell 2011). In addition, genomic locations where the DNA methylation level is upregulated by deletion of Tets are already identified using those mice. Using them should facilitate to clarify the importance of Tet enzymes.

This is another great suggestion. We agree that the knockout model of Tet2 would be an excellent way to test the requirement of Tet2 for OSK reprogramming. We avoided the whole-body Tet2 KO mice because they may have developmental defects. A better experiment was to obtain Tet2 conditional KO mice[43] (JAX:017573) that have normal RGCs to begin with, and then delete Tet2 in adult RGCs, with and without OSK treatment. We used a published CRE-AAV (Supplementary Fig. S3 of [27]) to remove exon 3 of Tet2, thereby removing the start codon of TET2 protein. We first validated CRE enzymatic activity in RGCs using a Tomato reporter mouse carrying a stop cassette flanked by *loxP* sites. When CRE AAV and +OSK AAV were co- infected, 95% of the OSK-positive cells had Tomato expression, indicating the knockout efficiency was around 95% (Extended Data Fig.6h). Importantly, the conditional knockout of Tet2 blocked OSK-mediated RGC survival and axon regeneration (Fig.2f and Extended Data Fig.6i, j). The data has been added to the manuscript and we thank the reviewer for the suggestion.

Fig. 2f



Extended Fig. 6 i, j



8. *There is a possibility that the promotion of regenerative capacity in RGCs by OSK is non-cell autonomous. Indeed previous studies have demonstrated that senescence is induced by forced expression of the reprogramming factors in vivo, and that those senescent cells enhance the cellular plasticity in neighboring cells in a non-cell autonomous manner through secretory components (Mosterio L et al., Science 2016, Chiche A et al., Cell Stem Cell 2017). Labeling OSK expressing cells by introducing a fluorescent protein together with OSK possibly addresses this problem.*

This is also a good point. We have multiple lines of evidence that the effect of OSK is cell- autonomous:

First, the data in the first submission strongly indicated a cell-autonomous effect on RGC survival. There are two types of cells in our experiments: those that ectopically express OSK and those that don't. If OSK influenced surrounding cells, one might expect that survival of OSK- and OSK+ cells would be similar. However, we found that the survival frequency of OSK- positive RGC was approximately three times that of nearby OSK negative RGCs, or GFP- positive and nearby GFP-negative RGCs in control sample (52% vs.15-21%), resulting in their relative abundance increasing post-injury from an initial 40% pre-injury to 70% post-injury (Extended Data Fig.4e-g).

Second, as suggested by the referee, we cloned the fluorescent mKate gene downstream of rtTA (rtTA-2A-mKate) in the dual AAV Tet-On system so we could monitor regeneration in the axon. In the OSK group, most surviving axons before the crush site were mKate positive, and those post the crush site were almost all mKate-positive along their axons. In contrast, in the control mice, only a minority of axons overlapped with mKate expression. These data are consistent with the effect being cell autonomous. (See Referee 2, point 8 for the data and the full response).

Third, to determine whether OSK acts directly on RGCs to promote regeneration, we injected AAV2-FLEX-tTA together with AAV2-TRE-OSK into the vitreous bodies of Vglut2-Cre and Vgat- Cre mice, resulting in RGC-specific and amacrine specific OSK expression, respectively (Extended Data Fig.4a-d). Our results show that OSK expression in RGCs, not in amacrine cells, induces robust RGC axon regeneration, further supporting the hypothesis that OSK acts on RGCs in a cell-autonomous fashion. (See Referee 4, point 7 for the full

response).

Minor concerns:

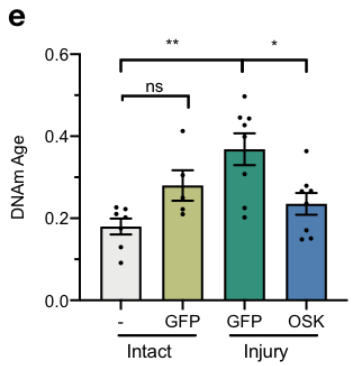
9. In Fig. 1b, the authors show that mice infected with OSK AAV have a survival rate comparable to wildtype mice. This is certainly an interesting observation, but this does not have any relevance to the main idea of the paper, or the relevance is not discussed. In addition, the following experiments expressing OSK are performed not in the whole body but in RGCs or in human cells. Therefore, this should go to a panel of Extended Figs.

Moved to Extended Data Fig.1i.

10. It seems that in some experiments decent controls are not prepared and/or not used for statistical comparisons. The followings are the examples: In Fig. 2e, RGCs expressing OSK before injury should be included. A statistical test should be done in the same "Injury" group.

Based on the results of our optic nerve crush experiments in Fig.2a-2d, where OSK treatment before injury did not promote regeneration, we did not include this group. Formally, it could be done of course, but the entire experiment would have to be done again at considerable cost and time, and it would be unlikely to provide additional information.

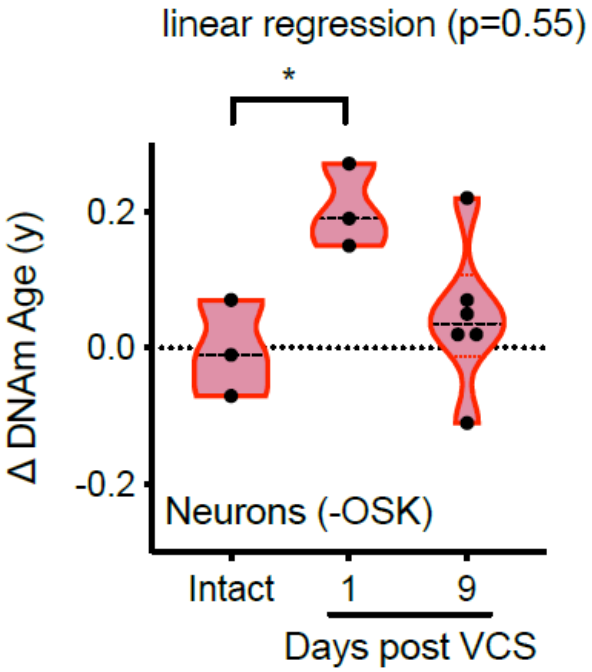
As suggested, we have added the statistical comparison to Figure 2e. Fig. 2e



11. In Fig.2h, DNAm Age of cells treated with VCS without OSK should be included in the analysis to demonstrate the decrease in DNAm Age is not due to the VCS treatment.

We have added this data to the manuscript as Extended Data Fig. 8d. There is a significant increase in DNAm Age at day 1 post VCS injury, consistent with acceleration of age after injury of the RGCs. These data indicate that the decrease in DNAm Age at Day 1 below baseline in Figure 2h is not due to VCS treatment.

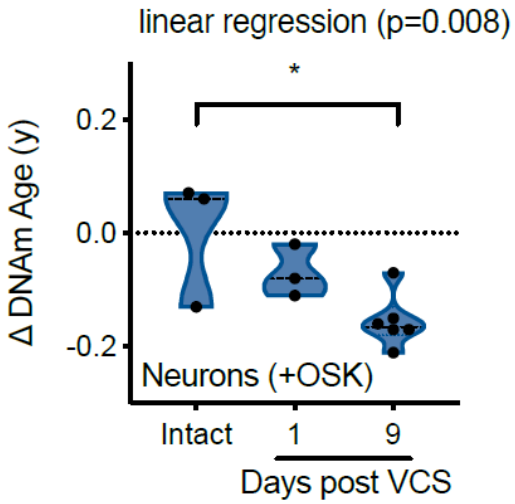
Extended Fig. 8d



12. Also, OSK expressing cells without VCS treatment should also be analyzed.

These data were originally included in Fig. 2h of the manuscript but they were not clearly labeled. We have revised the figure to make this clearer. See below; OSK expressing cells without VCS treatment is the “intact group”.

Fig. 2h



13. In Fig. 4i, the statistical comparison should be done between the sh-Scr sample and the sh- Tet1/2 samples under the same condition, e.g. +OSK. Plus, +OSK without shRNAs should be included, as in Fig. 4g and 4h. Otherwise the authors' conclusion that "Four weeks

of OSK AAV expression significantly decreased DNA methylation age" is not evidently supported by Fig. 4i.

We see the referee's point and, in fact, we had discussed this point as well in the manuscript. The issue is the complexity of the experiment, which involves isolating aged fragile RGCs by FACS, which takes 20 minutes per sample, during which the aged neurons need to be kept alive before sorting. It was, therefore, technically feasible to assess ~20 aged samples in a single experiment (21 samples were isolated, and this took 7 hours of FACS). Because the OSK sh-Scr group always behaves similarly to the +OSK in both the OMR and pERG assays, we decided the inclusion of these four groups were the best available option.

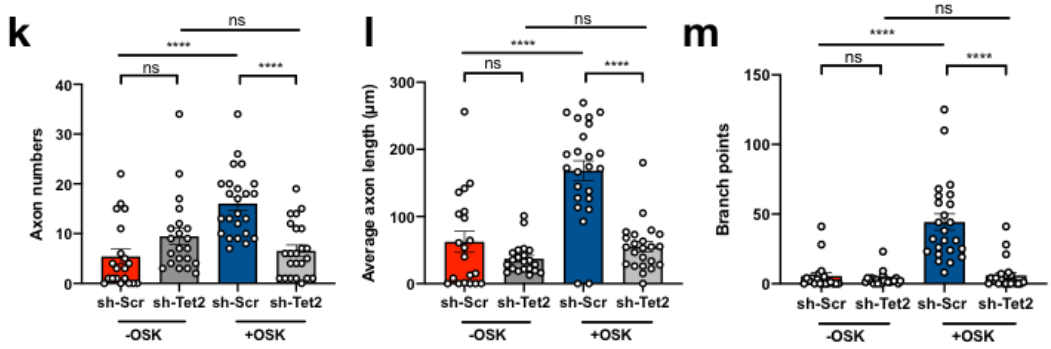
14. The authors suggest Tet dependent DNA demethylation have a role in the reprogramming process, but it is still not obvious from the experiments that Tets do not have a role in axon extension/regeneration itself. Seemingly only the comparison between the -OSK sh-Scr and - OSK sh-Tet2 in Fig 4i may address this point, which has no statistically significant difference. Can the authors demonstrate that Tet downregulation have an impact on the reprogramming process?

We agree. We compared Tet2 cKO (mediated by AAV-Cre) to Tet2 WT in the absence of OSK in the optic nerve crush model. We observed that knockout of Tet2 did not make any difference to RGC survival or to axon regeneration (Fig.2f and Extended Data Fig.6i, j).

15. In Fig. 2i and 2j, neurite area does not appear to be a right metric to assess axon extension capacity and amplifies differences to possibly lead to failure in evaluation (what if a neuron extends only a couple of very long neurite). Length of extended axons or some statistics of length such as mean or median seem to more accurately reflect the capacity.

As suggested, we quantified the axon numbers, average axon length and branch points, and included them as Extended Data Fig.8k-m (see below). The results are very similar to neurite areas (Fig. 2i).

Extended Fig. 8 k-m



Referee #4:

Comments to the Author

In this study, the authors showed that sustained expression of Oct4, Sox2, and Klf4 improve retinal ganglion cell survival and axon outgrowth in an acute nerve damage and glaucoma model in young and old mice. The effect sizes are sometimes high sometimes less pronounced (see points below). The effect was found to be Tet-dependent implying that DNA methylation plays a role. The strength of the paper is the apparently increased axon growth in various physical damage models of the retina or optic nerve. The weaknesses of the paper are (i) the somewhat unsatisfactory mechanism of what the 3 TFs actually do in retinal ganglion cells (if the mode of action is at all cell-autonomous)

Based on the referee's suggestion, we have greatly expanded the study to address the role of OSK, the role of DNA demethylation, and whether or not the effect is cell autonomous.

(ii) in particular in light of issues of novelty given previous reports that transient iPS cell reprogramming reverses aging-related phenotypes (Ocampo 2016).

We are the first to show that reprogramming can work ***after*** tissue damage has occurred. As Referee 1 states, the "*ability to enhance regeneration of already injured CNS axon at any point across the lifespan is exciting indeed*." In addition, we show that reprogramming can reverse the deleterious effects of ***normal*** ageing, can reverse the DNAme clock *in vivo*, that it can be done without causing death or sickness or inducing tumors, and that non-global, ***active*** DNA demethylation is required for the benefits of OSK, an *in vivo* mechanism that has not been shown previously in the context of reprogramming.

Moreover, from a practical point of view, I don't think that clinicians that read the paper would be now eager to inject 3 oncogenes into the retina

of glaucoma patients. Perhaps if the mechanism is better clarified there might be a more tractable therapeutic angle.

We beg to differ. Prompted by the referee, we tested if OSK expression increases the rate of tumorigenesis in mice over the course of a year of treatment, both in the eye and systemically. We found no induction of tumors in the eye (see Extended Data Fig. 2 c-i) or systemically (see Extended Data Fig. 1 j-n). In fact, clinicians, including those at the University of Pennsylvania Dept of Ophthalmology where the first gene therapies for the eye were developed are extremely excited about this technology. With their guidance, and those of other key opinion leaders, preparations for human trials to reverse blindness in glaucoma patients are underway. The fact that we don't see any safety issues in preclinical studies is what makes this finding so exciting.

It is worth noting how different our study is from previous ones[44,45]. Other studies used OSK + Myc while we used only OSK; (ii) the other studies relied on transgenic systems with permanent integration of the 4 factors causing leaky expression while we used a tight inducible AAV delivery method.

In summary, the paper describes an interesting phenomenon that is not well understood and has no direct therapeutic implication. Finally, the writing of the manuscript can be certainly improved, by omitting some far-fetched comparisons in the introduction and better describing the experimental details (see below).

Specific Points:

1. Authors should show the data about the risk of tumor formation induced by AAV-OSK expression.

We agree. We examined tumor formation after long-term AAV-OSK expression in the eye and systemically. In both cases, there was no increase in the tumor burden and the retinal structure remained intact (see Extended data 1j-o and Extended data 2c-i). Preparations for clinical trials are underway under the supervision of leading clinicians who are eager to test the technology in patients.

2. Knockdown of Tet 1/2 blocked the effects of OSK. Authors should also show the effect of knockdown of Tet 1/2 in axon density (Fig 3d), and RNA-seq pattern (Fig 4e, f).

This is a reasonable suggestion that we considered. We examined the knockdown of Tet 1/2 extensively in the acute nerve crush model, where axon density loss is greater than 80%. In glaucoma, however, the overall loss of axons observed in this short-term inducible model of glaucoma is minimal, so it's not as conducive to testing the effects of Tet1/2 knockdown.

Regarding assessing the effects on RNA-seq patterns, please see Referee 2 point 6.

3. If the mechanism is indeed simple DNA methylation, activation of Tet1/2 alone should promote axon regeneration and restore vision in these eye disease models.

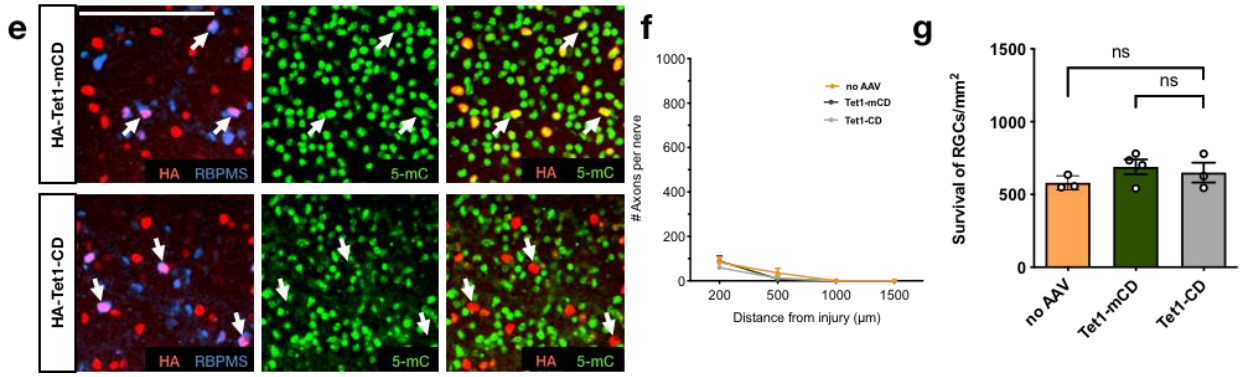
We may not have expressed our hypothesis clearly. We do not think the mechanism is simple DNA methylation globally. In support of this, we now find that OSK expression does not induce global changes in 5mC (see Extended Data Fig. 5 g-i). We hypothesize that OSK directs the Tets to specific loci to perform demethylation and now make this clear in the manuscript.
Whether this is a direct or indirect mechanism is not yet known but we are working on this as part of a follow-up paper.

Prompted by the reviewer, to rule out the possibility that DNA demethylation alone could promote axon regeneration *in vitro* and *in vivo*, we overexpressed the Tet1 catalytic domain and a catalytic mutant[38]. We could not overexpress the full-length Tet1 because it is over the AAV packaging limit. We observed that although the Tet1 catalytic domain decreased global DNA methylation (5mC), it did not provide any benefit to RGC survival and axon regeneration *in vitro*
or *in vivo* (see Extended Data Fig.7e-g and 8q).

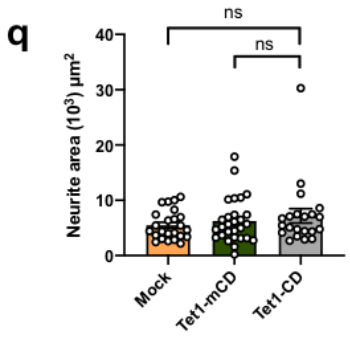
Taken together, Tet1/2 and the downstream gene TDG (required for *active* DNA

demethylation) are necessary but not sufficient for OSK to promote neuronal survival and axonal regeneration. This is supported by the fact that simple demethylation by overexpression of Tet has not been shown to induce iPSCs *in vitro* in the past 14 years of reprogramming work, as the initial demethylation steps for reprogramming need guidance.

Extended Data Fig. 7e, f, g



Extended Data Fig. 8q



4. The proposed model of the molecular action is underdeveloped. It is implied that the aging methylation marks play functional roles, but the correlations are quite weak and Tet knockdown should affect all DNA methylation, not just the aging-characteristic methylations.

We have improved our model as proposed in response above, and we have revised the manuscript to make this clearer. Our model, which is well supported by the data, shows that OSK treatment of RGCs does not induce replication-dependent DNAm dilution, a “passive” process. In contrast, the OSK-infected cells do not proliferate (Extended Data Fig.3a) and the benefits to the cells requires active DNA demethylation, as the new TDG KD experiments show (Extended Data Fig.7a-a). Consistent with this, OSK does not induce global changes to DNAm (Extended Data Fig.5g-i), but instead induces site-specific changes that are represented, in part, by the sites that constitute the DNAm clock. While the effect of OSK requires both Tets, we saw Tet knockdown didn’t cause a global increase in DNA methylation (Extended Data Fig. 5h, i) which would only be seen in proliferating cells but not in post-mitotic neurons like RGCs.

5. Fig. 1c: I don't see the controls: without infection, with infection but without dox, with control infection (tTA only e.g.).

The control infections are shown in Extended Data Fig.2b.

6. I would be curious to know how OSK were linked together and whether the 3 proteins are expressed at same ratios.

O,S and K are linked by 2A peptides (P2A and T2A) so the stoichiometry of expression is almost 1:1:1 (see Figure 1a).

7. Are the 3 TFs acting on retinal ganglion cell directly or are they causing other cells to change and secondarily influence axon outgrowth?

Great point. We have now examined this extensively and find that OSK works in a cell-autonomous fashion. The referee was correct that other cell types could be involved. To test this, we used two CRE transgenic mouse lines, Vglut2-CRE[2], in which CRE is selectively expressed in RGCs and Vgat-CRE, in which CRE is expressed selectively in inhibitory amacrine and horizontal cells, the other two cell types in the retina that can be transduced (Extended Data Fig.4a).

To determine whether or not OSK acts directly on RGCs to promote regeneration, we injected AAV2-FLEX-tTA together with AAV2-TRE-OSK into the vitreous bodies of Vglut2-Cre and Vgat- Cre mice, resulting in RGC-specific and amacrine specific OSK expression, respectively (Extended Data Fig.4b-d). Our results show that OSK expression in RGCs, not in amacrine cells, induces robust RGC axon regeneration (Fig.1g), further supporting the hypothesis that OSK acts on RGC in a cell-autonomous manner.

Extended Data Fig. 4 a-d

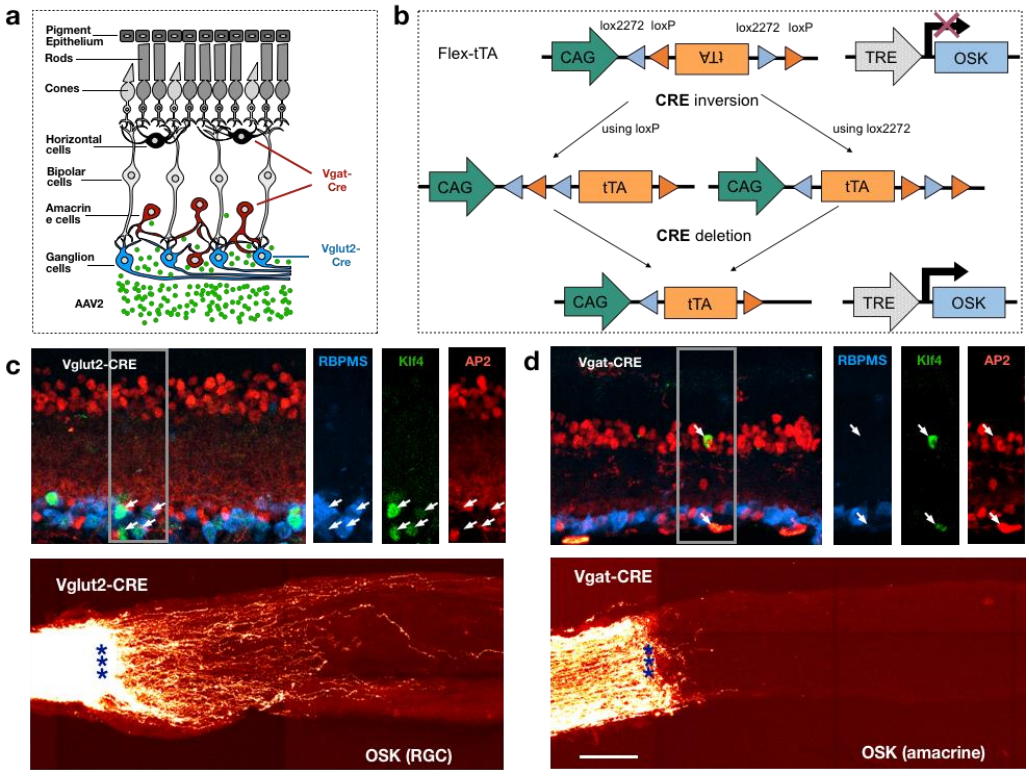
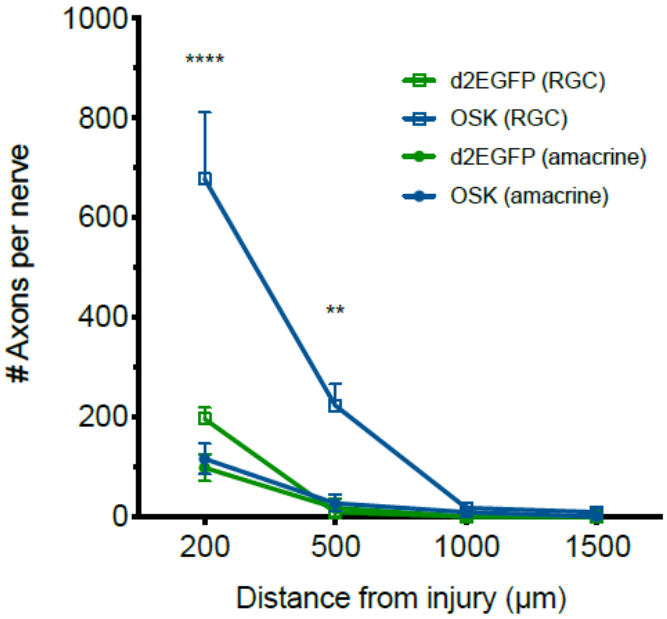


Fig. 1g



8. How long is the expression of the 3TFs needed?

In the optic nerve crush model, 4 weeks for young mice (Fig 1c), 7 weeks for old mice (Fig 4a). In the glaucoma model, 4 weeks (Fig 3a). In the ageing model, 4 weeks (Fig 4a).

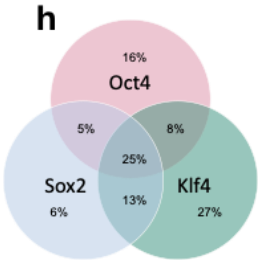
9. The text could be clarified and better aligned with the figures. E.g. In Fig1d the timing does not match what is described in the text. The CTB treatment shown in that Figure is not described in the text when it is first referred to. These things make it confusing and hard to read.

We have revised the text. The manuscript now reads, “we performed optic nerve crush in mice two weeks after delivering AAV2. After another two weeks, intravitreal injection of Alexa Fluor 555-conjugated cholera toxin subunit B (CTB), an anterograde axonal tracer, was performed to assess axon regeneration (Fig. 1c)”. Note the previous Fig.1d is now Fig.1c.

10. It further needs to be explained why the 3 factors delivered separately do not work, but the polycistronic vector does work. I don't see a quantification and statistical analysis in Suppl Fig. 3 that would explain the lack of an effect. At least one would expect to see a partial effect with the separate viruses.

Another great point. We quantified the infection rate for each of the 7 possible combinations of O, S, and K. The triple-infection frequency is only 25%, just as referee anticipated (Extended Fig. 3h, see below). The O+S+K group indeed has the second-best axon regeneration behind the OSK group (Fig.1d yellow line). It is worth considering that the stoichiometry of the three reprogramming factors may also contribute to efficacy. In the triple-infected cells, the stoichiometry of each factor will not consistent and this affects efficacy and quality, as shown in previous iPSC work[46]. We have clarified this in the text and added a new figure below.

Extended Fig. 3h



11. Another discrepancy is why the number of axons is ~5x higher but the number of ganglion cells only ~50% higher in OSK compared to the average of the remaining conditions. The # of ganglion cells and # of axons should be similar, if anything the # of axons should be lower because you might assume that not all cells extend axons. How much retrograde degeneration is happening? Is the effect thus mostly because of increased axon outgrowth or due to increased cell survival?

Improving cellular survival and axon regeneration are likely to be separate processes. Evidence supporting this possibility include the effects of Sox11 overexpression[1], which induces axon regeneration but decreases RGC survival. In our lab (Zhigang He's lab), we have found many treatments that improve RGC survival but do not improve axon regeneration. The number of axons is ~5x higher in OSK compared to the other groups but the number of ganglion cells is only ~50% higher. This indicates that the increased RGC survival only partially contributes to the increase in axon regeneration.

12. Confusing is also the timing of transgene expression. Fig.1d shows the transgenes were expressed first and then the lesion was induced. In Fig.2 the authors make the statement that transgene activation only after the lesion was helpful. Please clarify.

The confusion seems to have arisen because we use two different systems: (i) a Tet-Off and (ii) a Tet-On. In Fig. 1, we test the effect of OSK using the Tet-Off system to induce OSK before and after the damage. In Fig 2, we use the Tet-On system to show the beneficial effects of OSK do not require induction before damage.

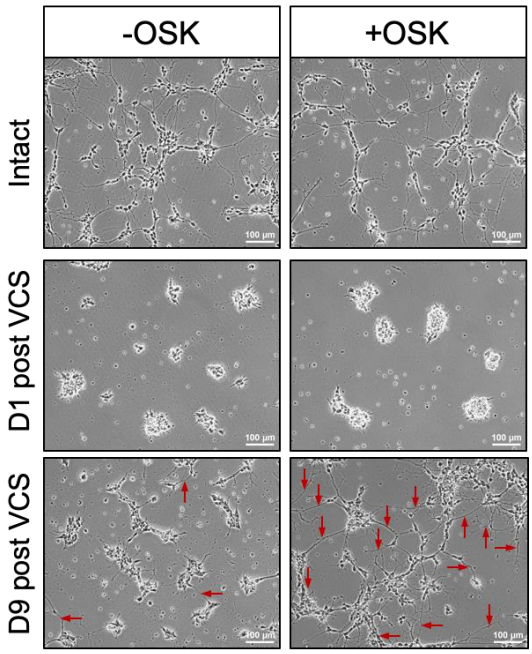
13. Please explain Fig. 1e: All 4 samples are from 1-month old animals? Does this mean that GFP infection dramatically increases the aging methylation signature? If this is true that OSK effects may have to be interpreted differently.

We presume the referee is referring to Fig.2e, where the groups are from 1-month old animals. The difference between intact and the GFP group that the referee mentions is not statistically significant by both One Way Anova, with posthoc ($p=0.15$) and t test ($p=0.08$), and the non- significant difference can be caused by the unavoidable injection procedure. Moreover, we have shown in human neurons that OSK continues to lower DNA methylation age post-injury. In these experiments, the controls are not GFP but undamaged OSK-infected neurons (Fig.2h).

14. The neuron outgrowth data in Fig 2j look weak. All neurons shown look very sick. Where are the positive controls? How do we know the culture conditions are permissive for healthy neuronal survival? Primary or human stem cell derived neurons look very different than what is shown. The rescue may seem large compared to the shown baseline but it is far from how normal neurons should look like.

The culture conditions are conducive for axonal regrowth. When observing an entire field, it is clear that there is robust growth of neurons in the untreated and in the recovered OSK-treated groups (See images below that are now included in Extended Data Fig. 8g). The growth doesn't look as robust in Fig. 2j because, in that particular experiment, cell clusters weren't split after damage, importantly to prevent cells from forming connections that would prevent us from quantifying the maximum axon regeneration that occurred without cell-cell contact.

Extended Data Figure 8g



15. Furthermore, it would be more accurate to measure the actual neurite lenghts and branchpoints rather than the area of neurites.

Great suggestion. We have now measured axon length and number of branch points and find that OSK also increases them after damage (Extended Figure 8k-m).

16. Use of human immortalized cell lines is not the state of the art anymore. Human ES or iPS cells provide a much better way to obtain human neurons.

We had considered these cells, too. But human neurons derived from ES or iPSCs are poor systems for studying ageing because their DNAm age is close to zero[31] and a youthful transcriptional profile is maintained[30]. See Referee #2, point 3.

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Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors responded to all my previous comments and concerns. The discovery of an intervention that can be performed *after* optic injury that restores or maintains visual function is important to emphasize. That is a distinguishing feature of this study.

One minor issue remains which is the mention and discussion of Goldberg et al., Science, which described down regulation of axon growth speed as a function of animal age. RGCs taken from embryonic rats maintained accelerated axon growth even as they aged several days to weeks and when placed into older host animals in vivo, arguing that extrinsic factors not merely clocks in the RGCs themselves were important. Nature papers are space limited for detailed discussion. As such I recommend citing these reviews (Nature. 2018 May;557(7705):359-367; Science. 2017 Jun 9;356(6342):1031-1034) which together address the context upon which the present results stand as a major step forward.

Summary after detailed re-review: an important study, timely and will be of exceptionally broad interest.

Referee #2 (Remarks to the Author):

The authors have made significant efforts to address previous concerns with some new data and clarification. Not all the questions have been addressed, but the manuscript is improved.

Remaining concerns from previous critics:
#2. I am not really satisfied the indirect answer by the authors.
#3. The results on SH-SY5Y should be removed. This is below the current standard in a high profile journal. And the same issue was raised by another reviewer.
#4. It will be important to know whether the effect is due to axonal regrowth or sprouting. Maybe the authors should some 3D whole-mouse clearing samples as examples to get a view of degree of sprouting.
#5. Because the authors can not show optomoter response behavioral test in the axonal crush model, the title of the manuscript is too strong and should be tuned down. There is no evidence of reversal of injury-induced vision loss, just axonal regeneration/sprouting.
#6. Give now single-cell or even single nucleus RNA-seq analyses have been very common, it is surprising that authors can not perform gene expression analysis as requested. These are important pieces of supporting evidence.

Other comment:
The authors should also tune down REVIVER and conclusion in the abstract. The paper provide evidence that axon regeneration can be initiated by reprogramming factors in several models and the process requires active DNA demethylation. The only data on epigenetic clock is a very low resolution analysis in Fig. 2e and there is no evidence that the reduction of methylation changes with OSK after injury requires Tet proteins. Without detailed gene expression and epigenomic analyses, there is very little evidence that "old tissues retain a faithful record of youthful epigenetic information that can be accessed for functional age reversal".

Referee #3 (Remarks to the Author):

The authors addressed most of my concerns. However, there remain some unanswered points which should be addressed before the publication.

The authors described that "non-global demethylation" is required but never showed the "specific demethylation". The rDNAm clock is just one readout of the Tet1/2-mediated DNA demethylation activity which is associated with the reversal. However, this reviewer feels that most descriptions throughout the manuscript can easily mislead readers as if the authors have identified the "aging clock" that has a role in the aging- and damage-related phenotypes, which could be reversed by OSK expression.

Again, it is interesting if the authors could identify loci exhibiting increased DNA methylation in aging- and damage-related phenotypes in an unbiased manner, which hopefully include rDNAm clock, and such increased DNA methylation levels can be reduced by the transient OSK expression. It is also important that the reversal effects are sustained even after the withdrawal of OSK, which excludes the possibility that exogenous OSK expression directly leads to the reversal phenotypes. Although the authors provided the data in the rebuttal, this should be included in the manuscript because "epigenetic information" is the central message of this manuscript.

Collectively, unless the authors demonstrate causal relationship of the rDNAm clock or identify the specific loci that are epigenetically involved in the reversal, I believe that the authors should substantially revise the text and need to tone down statements with regard to the aging clock and epigenetics.

REFEREE#2 for REFEREE#4
In response to reviewer 4's comments, the authors performed some new experiments to address some of the concerns. Reviewer 4 has two general comments, one on the novelty and mechanistic insight and the other on whether the mode of action is cell autonomous. For the first concern, the authors pointed out that they observed axon regeneration after injury, which is different from previous models, thus quite significant and they also provided new evidence for the involvement of active DNA demethylation as a mechanism. The concern for whether the effect is cell autonomous is partially addressed and can be better examined (See #7).
Original specific concerns:
#1. The authors provided data on the safety of OSK manipulation, showing a lack of tumorigenesis.
#2. The authors provided data on axonal density, but not RNA-seq analysis, which is also requested by another reviewer.
#3. The author provided new data that upregulation of Tet1 demethylation activity is not sufficient for regeneration. The authors should be very specific in the text that active DNA demethylation is required, but not sufficient for OSK-induced axon regeneration after injury, which also has been shown in other models of axon regeneration (PMID: 28426967).
#4. The argument is fine, although the authors did not show the aging mark changes are blocked by Tdg manipulation.
#5. Fine.
#6. Fine.
#7. The experiment done in response to the critic does not rule out the non cell autonomous contributions. The better experiment is to examine whether there is any regeneration of axons in cells that do not express OSK.
#8. Ok.
#9. OK.
#10. OK.
#11. The explanation is not satisfactory. It could well be due to sprouting of axons, which the authors should examine and show more carefully and clearly state it in the text.
#12. OK
#13. OK
#14-16. The same caveat of using tumor cell lines has been raised by other reviewers and the

data should be removed.

REFEREE#3 for REFEREE#4
Regarding the authors response to Reviewer #4, I feel that the authors adequately answered most concerns. I agree that this study is not enough to ensure the safety of this strategy for the clinical application (related to specific point 1). However, I don't think this is the main message of this study, and future preclinical studies should solve this issue. A precise mechanism for the increased axon growth, except for the involvement of Tet enzymes, is still missing here. Especially, it is not clear if the altered DNA methylation at specific loci indeed plays a role in the axon growth (specific point 3 and 4). However, given that we still do not know a precise mode of action of Yamanaka factors in most consequences after OSK induction, which include reprogramming, rejuvenation and cancer development, it seems very difficult to uncover the mechanism beyond the involvement of Tet enzymes. Still, I think the striking phenotypes described here should open up novel strategies for axon regeneration, which could be a potential therapeutic approach in a wide range of diseases. Overall, I think that the authors answered all concerns from reviewer #4 which can be addressed in this setting.

Author Rebuttals to First Revision:

Nature 2019-07-11416B

We were pleased the referees found our revised manuscript "important," "timely," with "novel strategies" and "striking" findings "of exceptionally broad interest," with technology that "could be a potential therapeutic approach in a wide range of diseases."

We have toned down statements where suggested, reformatted the figures, and trimmed the text to meet *Nature*'s guidelines. Importantly, based on reviewers' advice, we have now determined the effect of injury and ageing on DNA methylation, and the ability of OSK to counteract these changes on a genome-wide level. To help analyze the data, we recruited another leader in the DNA methylation field, Dr. Morgan Levine from Yale. The work has led to some interesting additional findings:

1. The effect of injury on DNA methylation parallels the effect of ageing ($p < 1e^{-200}$).

2. A set of 1,226 CpG sites that change with injury and ageing are substantially reset by OSK.

3. These CpGs are enriched at genes involved in synaptic/neuronal processes and associate with binding sites for PRC2 and its product, H3K27me3, both of which are involved in maintaining stem cell plasticity and are associated with DNAm clock sites.

We are excited about how much better the paper is after the two rounds of revisions, and hope the referees find this version suitable for publication.

Referee's comments are in blue italics.

REFEREE 1

*The authors responded to all my previous comments and concerns. The discovery of an intervention that can be performed *after* optic injury that restores or maintains visual function is important to emphasize. That is a distinguishing feature of this study.*

Thank you. We agree and have now emphasized this feature.

One minor issue remains which is the mention and discussion of Goldberg et al., Science, which described down regulation of axon growth speed as a function of animal age. RGCs taken from embryonic rats maintained accelerated axon growth even as they aged several days to weeks and when placed into older host animals in vivo, arguing that extrinsic factors not merely clocks in the RGCs themselves were important. Nature papers are space limited for detailed discussion. As such I recommend citing these reviews (Nature. 2018 May;557(7705):359-367; Science. 2017 Jun 9;356(6342):1031-1034) which together address the context upon which the present results stand as a major step forward.

We have included the two references.

Summary after detailed re-review: an important study, timely and will be of exceptionally broad interest.

REFEREE 2

The authors have made significant efforts to address previous concerns with some new data and clarification. Not all the questions have been addressed, but the manuscript is improved.

Remaining concerns from previous critics:

#2. I am not really satisfied the indirect answer by the authors.

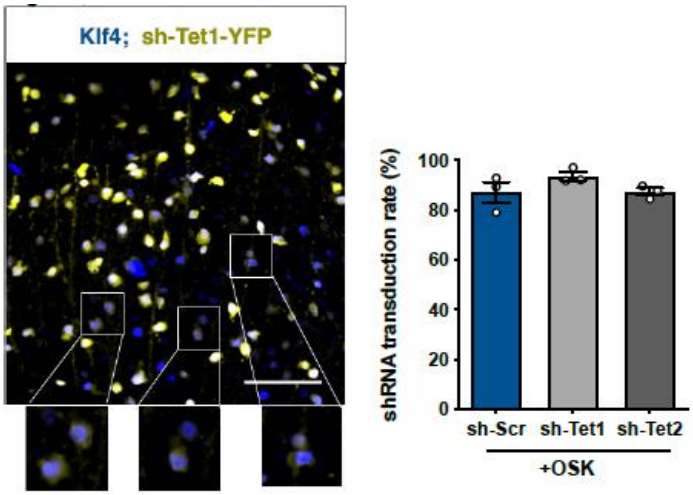
"Given only 70% overlap of shRNA-Tet1 or Tet2 expression with OSK vectors, you would expect still see significant number of regenerating RGC axons. But the results showed no axons longer than 500 µm (Fig. 2f)."

In the first submission, we were ultra-conservative in our estimates of infection. Only strongly YFP-positive cells were counted. This gave the figure of 70% YFP-Klf4 overlap. Based on the referee's comment, and upon further consideration, we believe it is more appropriate to include weaker YFP-positive cells as well (see images below). We find the co-localization is now 90% (new Extended Fig.6c). In the image below, for example, the overlap went from 75.8% to 97%.

To test Tet2-dependence in a different way and bolster this result, we have now induced

OSK in a Tet2 conditional KO and found that it abolished the effect of OSK on both axon regeneration and cell survival (Fig. 2f and Extended Data Fig. 6h-j), further supporting our original interpretation.

Fig. 6b, c

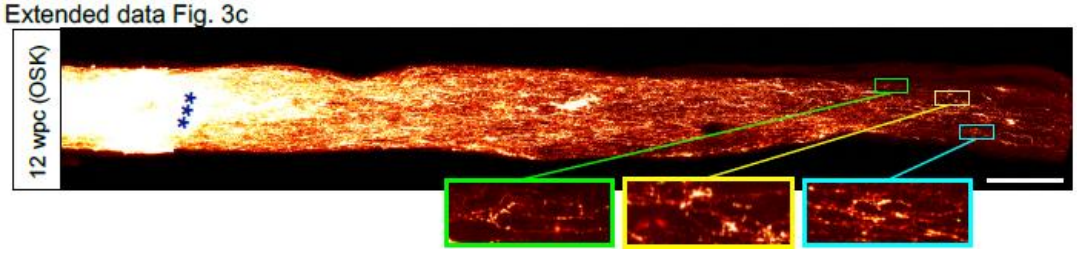


#3. The results on SH-SY5Y should be removed. This is below the current standard in a high profile journal. And the same issue was raised by another reviewer.

We have removed the SH-SY5Y results from the main figures.

#4. It will be important to know whether the effect is due to axonal regrowth or sprouting. Maybe the authors should some 3D whole-mouse clearing samples as examples to get a view of degree of sprouting.

Extended data Fig. 3c



As suggested, we have applied whole-nerve clearing technology in the nerve crush experiment, now presented in Extended data Fig. 3c. Local axonal sprouting is observed distal to the injury site (see insets). Importantly, this technique only works when the axon density is low. When the density is high, such as before the crush site (left side, above), it's not feasible to see individual axons and sprouting.

In the glaucoma model, RGC axons degenerate gradually in response to elevated intraocular pressure (IOP), with a maximum loss of only 25-30% of axons and a much higher axon density compared to the optic nerve crush model. So, while this technique works well for identifying axon sprouting in the optic nerve crush model, it is not suitable

for identifying axon sprouting in the glaucoma model.

We agree that showing axon sprouting in our glaucoma model would be interesting. These data, however, are not critical to the paper and would not affect our conclusion that OSK can reverse vision loss *after* glaucomatous damage has already occurred. Future studies in our labs will attempt to solve the technical problems of detecting axon sprouting in the glaucoma model using 3D reconstruction of serial transmission electron microscopy (TEM) sections through the optic nerve head, which will be an enormous amount of work and is beyond the scope and timeline of this present study.

#5. Because the authors can not show optomoter response behavioral test in the axonal crush model, the title of the manuscript is too strong and should be tuned down. There is no evidence of reversal of injury-induced vision loss, just axonal regeneration/sprouting.

Sorry, we were not clear. By "injury" we meant glaucoma-induced vision loss, not the crush model. To avoid confusion of readers and meet the title character limit, we've removed the word "injury".

#6. Give now single-cell or even single nucleus RNA-seq analyses have been very common, it is surprising that authors can not perform gene expression analysis as requested. These are important pieces of supporting evidence.

We agree single cell RNA-seq will be useful, given the amount of material we are able to collect in the Tet knockdown experiment is insufficient for bulk-seq, which is why we used qPCR. That said, bulk-seq has distinct advantages over single cell RNA-seq in experiments involving retinal ganglion cells. One of our labs (Zhigang He's) and Josh Sane's lab have identified at least *46 types* of RGCs using single cell RNA-seq and it would be extremely challenging and to interpret data with 46 different cell types (Tran et al. Neuron, 2019)[1]. Due to technical challenges with single cell experiments in general, only changes to high-abundance transcripts are detectable, especially in samples as tiny as sorted mouse retinal ganglion cells. As revealed by our bulk-seq results, genes that change during aging are mostly expressed at low levels (Fig. 4c heatmap), making bulk-seq the method of choice for these experiments. As the technology improves, it may be possible to use single-cell RNA-seq to quantify low-abundance transcripts and dissect out the effects of OSK and Tet knockdowns among the 46 types of RGCs.

#7 Other comment:

The authors should also tune down REVIVER and conclusion in the abstract. The paper provide evidence that axon regeneration can be initiated by reprogramming factors in several models and the process requires active DNA demethylation. The only data on epigenetic clock is a very low resolution analysis in Fig. 2e and there is no evidence that the reduction of methylation changes with OSK after injury requires Tet proteins.

Without detailed gene expression and epigenomic analyses, there is very little evidence that "old tissues retain a faithful record of youthful epigenetic information that can be accessed for functional age reversal".

As suggested, we have removed “REVIVER” throughout the manuscript.

We have also performed genome-wide analyses of DNA methylation in both the crush injury and ageing experiments. We find that injury alters DNA methylation patterns in a way that is highly correlated with aging ($p < 1e^{-200}$) (see new Extended Data Fig. 5i below) and OSK reverses these DNA methylation changes (see new Fig. 2e below). In further support of DNAm playing a functional role in the effects of OSK, gene ontology (GO) analysis of these CpG sites found the top associated processes (9/10) with neuronal functions including synaptic transmission and the sensing of light, which are expected to get impaired post crush injury (Extended Data Fig. 5k).

To gain insights into how DNA methylation changes might be involved in the effects of OSK, we used machine learning to identify a methylation ageing signature based on 1,226 sites from genome-wide DNAm datasets, derived from the first principal component (PC1) in multi-age training samples, standardized to have a mean=0 and s.d.=1 (Supplementary Table 3, Extended Data Fig 10e). In an independent set of test samples, OSK reversed the DNAm ageing signature in the context of both injury and ageing (Fig. 4e and Extended Data Fig 10f). Interestingly, these signature CpG sites were associated with genes involved in synaptic and neuronal processes, and regions enriched for the polycomb repressive complex 2 (PRC2) and its histone methyltransferase product, H3K27me3, both of which are known to recruit Tet enzymes[2,3] (Extended Data Fig 10g-i). Knockdown of either Tet1 or Tet2 altered the methylation status of the signature CpG sites in a similar way ($r = 0.4$, $p = 2.53e^{-45}$) and counteracted the effect of OSK on DNAm age (Extended Data Fig. 10j-l). Combined with our previous result that both Tet1 and Tet2 are required to improve RGC function and restore vision in 12-month-old mice, these data indicate changes to DNA methylation patterns may play a functional role in the benefits of OSK.

To avoid any confusion, we have added the sentences: “That said, we do not wish to imply that DNA methylation is the only epigenetic mark involved in this process. It likely involves other transcription factors and epigenetic modifications, such as the PRC2 complex and H3K27me3, both of which are involved in maintaining stem cell plasticity and are associated with DNAm clock sites[4-6].”

Extended Data Fig. 5i

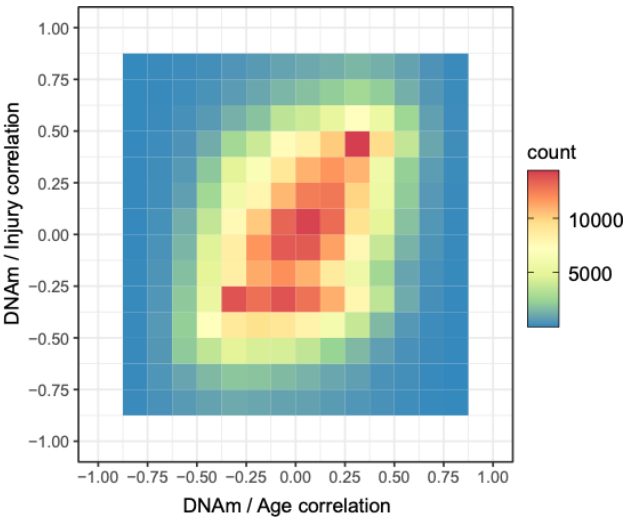
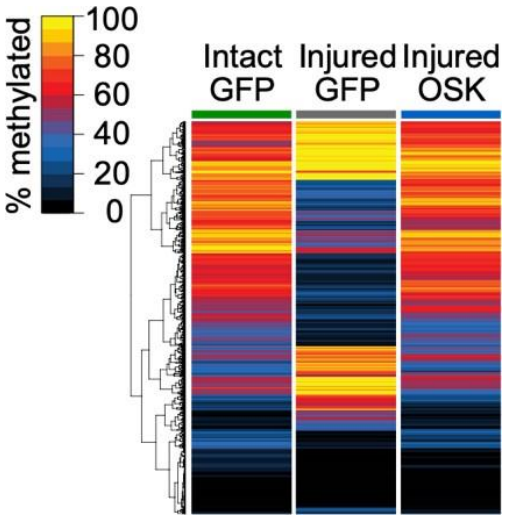
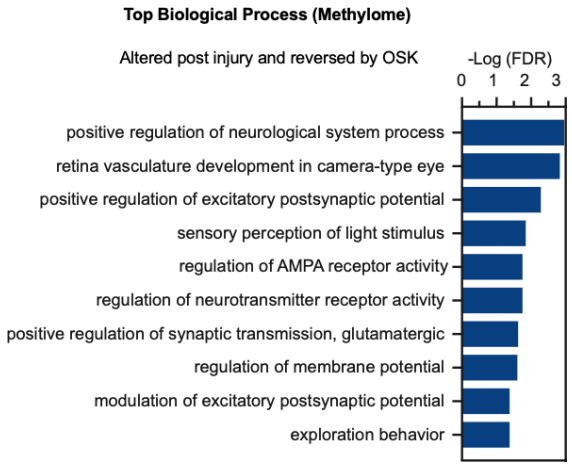


Fig. 2e



Extended Data Fig. 5k



Extended Data Fig. 10e

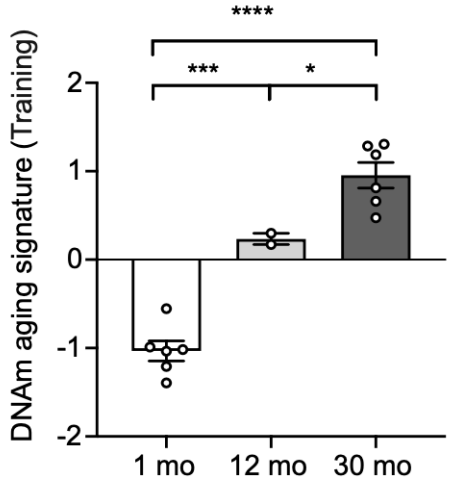
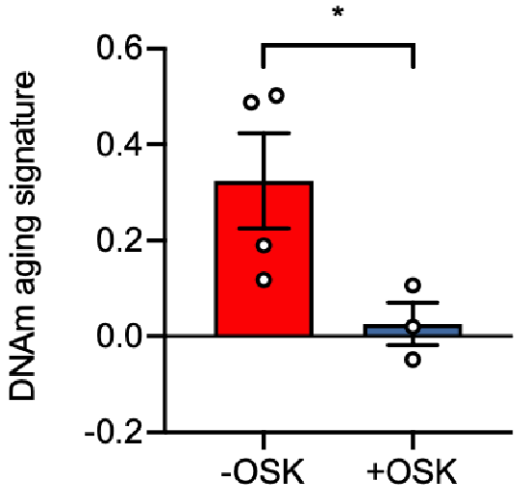
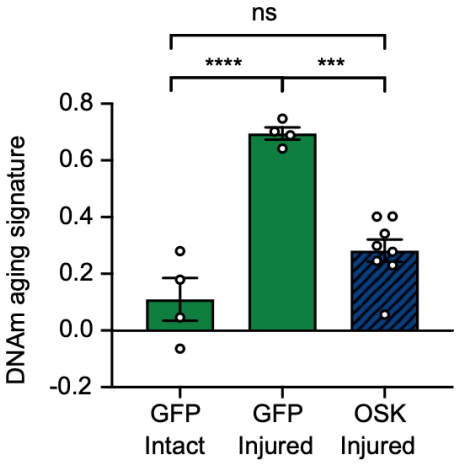


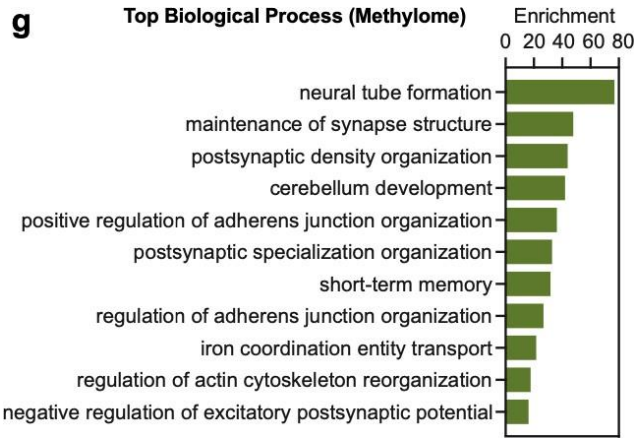
Fig. 4e



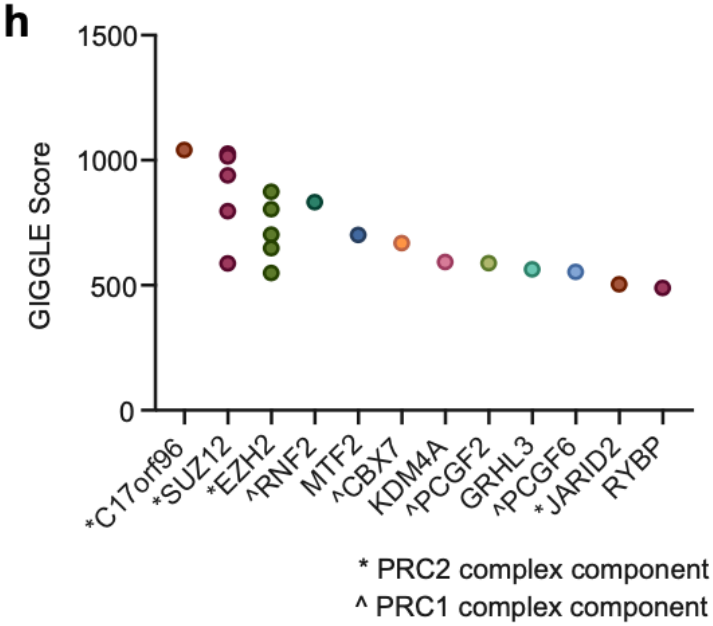
Extended Data Fig. 10f



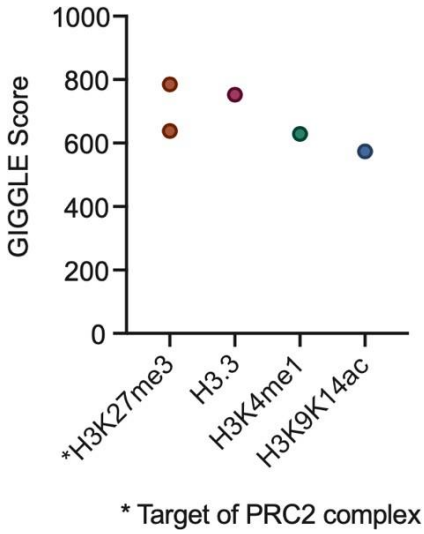
Extended Data Fig. 10g



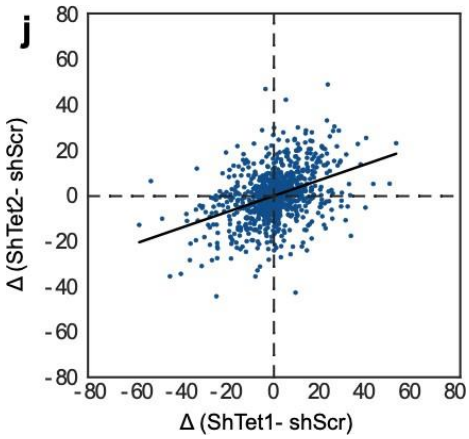
Extended Data Fig. 10h



Extended Data Fig. 10i



Extended Data Fig. 10j



REFEREE 3

The authors addressed most of my concerns. However, there remain some unanswered points which should be addressed before the publication.

The authors described that "non-global demethylation" is required but never showed the "specific demethylation". The rDNAm clock is just one readout of the Tet1/2-mediated DNA demethylation activity which is associated with the reversal. However, this reviewer feels that most descriptions throughout the manuscript can easily mislead readers as if the authors have identified the "aging clock" that has a role in the aging- and damage-related phenotypes, which could be reversed by OSK expression.

We completely agree that the rDNA clock is not the reason for rejuvenation. We did not mean to imply it. Based on the excellent suggestion, we have analyzed the DNA methylome. See Referee 2 point #7. We have also clarified the text to make it very clear that genome-wide changes to DNA methylation pattern, not a specific DNAm clock, are likely to be involved in the effects of OSK.

Again, it is interesting if the authors could identify loci exhibiting increased DNA methylation in aging- and damage-related phenotypes in an unbiased manner, which hopefully include rDNAm clock, and such increased DNA methylation levels can be reduced by the transient OSK expression. It is also important that the reversal effects are sustained even after the withdrawal of OSK, which excludes the possibility that exogenous OSK expression directly leads to the reversal phenotypes. Although the authors provided the data in the rebuttal, this should be included in the manuscript because "epigenetic information" is the central message of this manuscript.

This was a great suggestion. By analyzing genome-wide CpGs covered by uniquely mapped reads, we find that OSK reverses the effects of injury and ageing on DNA methylation at 1,226 CpGs (Supplementary Table 3). We have found strong associations between these sites and synaptic/neuronal processes. We also found their associations with polycomb repressive complex 2 (PRC2) binding sites and its histone methyltransferase product, H3K27me3, both which are known to recruit Tet enzymes. These findings are now presented in the paper. Again, see Reviewer 2, point #7.

We agree it is exciting that the effects of OSK last for at least two weeks. In fact, we are performing much longer experiments to see how long the effect lasts. Not only are these experiments going to take at least another 9 months to complete with multiple replicates, they are planned as part of another study with another lab, so we hope the reviewer can appreciate why we are unable to include them in this manuscript. Given that: (i) we have shown that active DNA methylation is involved in the effects of OSK (requiring Tets and TDG); (ii) that DNA methylation is known to be a long-lasting epigenetic change; (iii) that we now have associations with the CpGs altered by OSK and the PRC2 complex and H3K27me3, we hope referee will allow us to refer to the effects of OSK as epigenetic.

Collectively, unless the authors demonstrate causal relationship of the rDNAm clock or identify the specific loci that are epigenetically involved in the reversal, I believe that the authors should substantially revise the text and need to tone down statements with regard to the aging clock and epigenetics.

Please see responses above. We have substantially toned down and clarified the text: "That said, we do not wish to imply that DNA methylation is the only epigenetic mark involved in this process. It likely involves other transcription factors and epigenetic modifications, such as the PRC2 complex and H3K27me3, both of which are involved in maintaining stem cell plasticity and are associated with DNAm clock sites[4-6]."

REFEREE#2 for REFEREE#4

In response to reviewer 4's comments, the authors performed some new experiments to address some of the concerns. Reviewer 4 has two general comments, one on the novelty and mechanistic insight and the other on whether the mode of action is cell autonomous. For the first concern, the authors pointed out that they observed axon regeneration after injury, which is different from previous models, thus quite significant and they also provided new evidence for the involvement of active DNA demethylation as a mechanism. The concern for whether the effect is cell autonomous is partially addressed and can be better examined (See #7).

We agree the ability to regenerate RGCs after injury is a significant result. Thank you.

Original specific concerns:

#1. The authors provided data on the safety of OSK manipulation, showing a lack of tumorigenesis.

#2. The authors provided data on axonal density, but not RNA-seq analysis, which is also requested by another reviewer.

#3. The author provided new data that upregulation of Tet1 demethylation activity is not sufficient for regeneration. The authors should be very specific in the text that active DNA demethylation is required, but not sufficient for OSK-induced axon regeneration after injury, which also has been shown in other models of axon regeneration (PMID: 28426967).

We have now made it very clear in the text that "These data indicate that active DNA demethylation is necessary for OSK, but not sufficient by itself, to protect RGCs and regenerate axons.".

#4. The argument is fine, although the authors did not show the aging mark changes are blocked by Tdg manipulation.

Thanks for the comment. We have been very specific in the text that active DNA demethylation is necessary for OSK-induced axon regeneration after injury.

#5. Fine.

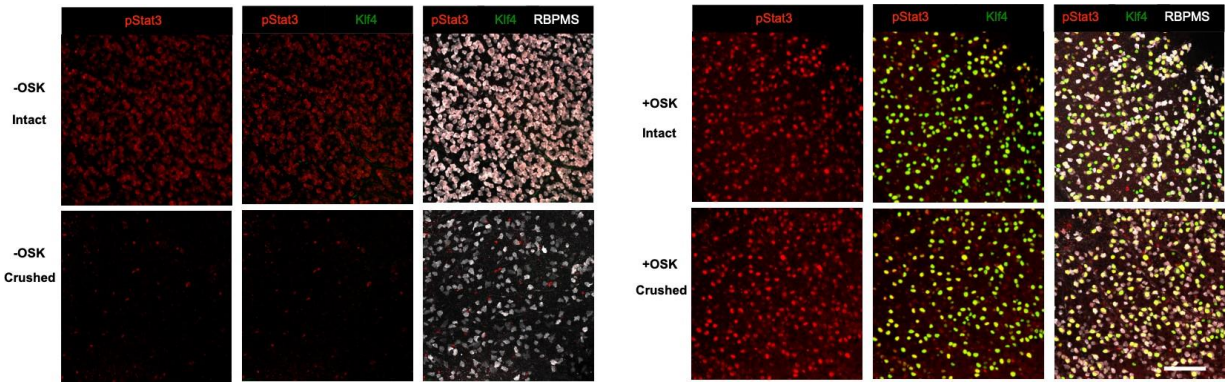
#6. Fine.

#7. The experiment done in response to the critic does not rule out the non cell autonomous contributions. The better experiment is to examine whether there is any regeneration of axons in cells that do not express OSK.

Because amacrine cells have been recently reported to secondarily affect RGC axon

growth[7], we interpret referee 4's original question being whether the effect is directly on RGCs or another cell type that AAV2 can infect. This was addressed using Vgat-CRE transgenic mice that allowed is to induce OSK in amacrine cells. Our other experiment, inspired by Referee 2 in the previous revision, showed that pStat3 is upregulated exclusively in OSK-positive RGCs, indicating a cell-autonomous mechanism within the RGCs for axon regeneration. We agree with Referee 2 that our experiments do not completely rule out the non-cell autonomous contributions, so we now carefully avoid any statement that would indicate that axon regeneration is fully cell autonomous.

Extended Fig.5a



#8. Ok.

#9. OK.

#10. OK.

#11. The explanation is not satisfactory. It could well be due to sprouting of axons, which the authors should examine and show more carefully and clearly state it in the text.

We now show detailed images of sprouting in Extended Data Fig. 3c. As suggested, for clarity, we have added the sentence: "Indeed, when polycistronic OSK was induced for 12-16 weeks, regenerating and sprouting RGC axon fibers extended over 5mm into the chiasm, where the optic nerve connects to brain."

#12. OK

#13. OK

#14-16. The same caveat of using tumor cell lines has been raised by other reviewers and the data should be removed.

We have moved the human neuron data from the main figures into Extended data.

REFEREE#3 for REFEREE#4

Regarding the authors response to Reviewer #4, I feel that the authors adequately answered most concerns. I agree that this study is not enough to ensure the safety of

this strategy for the clinical application (related to specific point 1). However, I don't think this is the main message of this study, and future preclinical studies should solve this issue.

We appreciate it.

A precise mechanism for the increased axon growth, except for the involvement of Tet enzymes, is still missing here. Especially, it is not clear if the altered DNA methylation at specific loci indeed plays a role in the axon growth (specific point 3 and 4). However, given that we still do not know a precise mode of action of Yamanaka factors in most consequences after OSK induction, which include reprogramming, rejuvenation and cancer development, it seems very difficult to uncover the mechanism beyond the involvement of Tet enzymes.

We thank referee for understanding the difficulty of such a task. That said, we have made progress since the last revision in identifying putative players. Our new analyses of DNA methylation at genomic CpGs strongly point to the involvement of the epigenetic regulatory complex PRC2 and its product, H3K27me3, both of which are known to recruit DNMTs and Tets and are associated with DNAm clock sites. Future work by our lab and others will determine if PRC2 and changes in H3K27me3 are necessary for the effects of OSK *in vivo*.

Still, I think the striking phenotypes described here should open up novel strategies for axon regeneration, which could be a potential therapeutic approach in a wide range of diseases. Overall, I think that the authors answered all concerns from reviewer #4 which can be addressed in this setting.

We appreciate the positive comment, and greatly thank the referees for their expertise and extremely helpful suggestions, especially during this difficult time.

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