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miR-24 and its target gene Prdx6 regulate viability and senescence of myogenic progenitors during aging

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13th July 2021

Dear Prof Antebi,

Thank you for the initial assessment of our manuscript (ACE-21-0072) and the reviewer comments sent on the 6th of April. Clarification of the role of Prdx6 in satellite cells ageing in addition to its characterisation as miR-24 target gene was requested. We have now repeated all the experiments performed in the first version of this manuscript using miR-24 mimic and inhibitor with the addition of another condition: cell transfected with Prdx6 siRNA. Several key reagents, such as miR-24 inhibitor or Prdx6 siRNA have been discontinued (indicated in supplementary data) since we collected original data, therefore we decided to repeat all experiments. We have included additional analyses of mitochondrial ROS, mitochondrial morphology, DNA damage and additional assays of senescence.

The results obtained confirm our previous findings that both treatment with miR-24 mimic and downregulation of Prdx6 using siRNA resulted in reduced viability, decreased myogenic potential and increased senescence during aging. In summary, our results identify a role for miR-24-3p through inhibition of Prdx6 in satellite cells during aging which may play a key role in early stages of skeletal muscle regeneration after acute injury, through controlling adaptive redox, apoptotic and senescence signalling pathways. Moreover, our findings show that miR-24 and Prdx6 regulation of myogenic progenitor phenotype is more pronounced in cells from old mice, likely due to miR-24 regulation of additional to Prdx6 target genes, such as p21.

Considering the number of additional experiments that were performed we propose joint first name authorship to the first two authors (Soriano-Arroquia and Gostage). We also had significant input for a number of experiments from PhD candidate Qin Xia and believe their contribution warrants authorship on the manuscript. We believe the additional experiments and overall editing have significantly improved the manuscript and it could now be re-considered for publication in *Aging Cell*.

We hope you will find the revised article appropriate for publication in *Aging Cell* and look forward to your reply.

Yours sincerely,

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ACE-21-0072 *miR-24:Prdx6 interactions regulate oxidative stress and viability of myogenic progenitors during aging*

Response to reviewer comments

Editor comments:
As you can read below, the reviewers and supervising editor were interested in the possible functional interaction of mir-24/prd6, but felt that that several points about this interaction with respect to aging needed clarification, as well as the relationship to ROS. Furthermore, the statistical analysis needs to be addressed.
The review of your manuscript is complete, and the reviewers found the manuscript of interest, relevant for Aging Cell and will appeal to a wide readership. The reviewers ask for revisions of the manuscript requiring more details regarding the use of t-tests for statistical significance, resolution of apparent inconsistencies among experiments, particularly with regard to the effects of miR24 antagomiRs and miR24 in aged and young mice.

We would like to thank the reviewers and the editors insightful comments and an opportunity to improve this manuscript. We have addressed all the comments – please see below.

The reviewers and editor requested clarification of the role of Prdx6 in satellite cells ageing in addition to its characterisation as miR-24 target gene. We have now repeated all the experiments performed in the first version of this manuscript using miR-24 mimic and inhibitor with the addition of another condition: cell transfected with Prdx6 siRNA. Several key reagents, such as miR-24 inhibitor or Prdx6 siRNA have been discontinued (indicated in supplementary data) since we collected original data, therefore we decided to repeat all experiments.

All statistical analyses have been performed on raw values; T test have only been used for pairwise comparison if data was deemed normally distributed, otherwise Mann Whitney test was used. For multiple comparisons, we used One Way A.N.O.V.A followed by Tukey's multiple comparison or Kruskal Wallis test followed by Dunn's multiple comparison, as indicated in the figures.

We are happy to note that between the two different experimental settings and two sets of different reagents: miR-24, AM24 and siPrdx6, all data is consistent in terms of the phenotype elucidated by miR-24 and Prdx6. We included original data in the supplementary figures for the purpose of transparency and further support of the conclusions.

We believe the differences in the senescence phenotype between adult and old cells in the original data may results from a wide range of ages of mice used for myogenic progenitor isolation, e.g. cells from adult mice were isolated from mice 1-8 months old and cells from old mice were isolated from mice aged 20-24 months old. These ages are associated with dynamic changes in muscle, therefore we isolated myogenic progenitors from mice aged 6 (adult) or 24 (old) months old mice for the experiments contained within the revised manuscript.
The phenotype presented in the revised manuscript is consistent between adult and old cells with miR-24 having a more pronounced effects in cells from old mice; this is not entirely surprising as miR-24 has been previously shown to regulate senescence- and cell cycle associated genes which are dysregulated during ageing (please see page 9, discussion). Moreover, our data presented in the revised manuscript clearly demonstrate that downregulation of miR-24 target gene, Prdx6, is associated with disrupted mitochondrial morphology, increased mitochondrial ROS generation, increase in the levels of DNA damage marker: phosphorylated H2AX and consequently decreased cell viability, myogenic potential and increased senescence. In addition to determining the role of Prdx6 in satellite cells during ageing, we propose a mechanism by which miR-24 and Prdx6 changing

levels regulate ROS homeostasis and cell viability, senescence and myogenic potential during ageing. Please see below for the detailed response to the reviewer comments.

In addition, both reviewers ask for clarification and additional experiments solidifying the data regarding ROS and the regulation of ROS by miR24 and Prdx6. The supervising editor agrees with the reviewers' assessments and believes that additional data supporting a role for Prdx6 in regulating senescence would support the conclusions as no effects of Prdx6 manipulation on satellite cell senescence were provided.

Thank you for this suggestion. We have repeated all the previous cell experiments presented in the original manuscript using young and old mouse myogenic progenitors and human myogenic progenitors, treated with either scrambled control, miR-24, antagomiR-24 but also including an additional group with siPrdx6 to further elucidate the role of Prdx6 in myogenic progenitor viability, myogenic potential and senescence. These results are further complimented with additional experimentation including detection of mitochondrial ROS using Mitosox Red, quantification of DNA damage repair through immunostaining for phosphorylated H2AX, Western blots for Prdx6, Westerns for another mitochondrial peroxidase Prdx3 and the chaperone protein Hsp70.

Reviewer 1

Thank you for the invitation to review "miR-24: Prdx6 interactions regulate oxidative stress and viability of myogenic progenitors during aging" by Soriano-Arroquia et al. In this manuscript the authors provide mechanistic insight, spanning cells models, animal models and primary human tissue, to the interaction of miR24 and peroxiredoxin-6 and satellite cell function in the context of skeletal muscle ageing. The manuscript is very well written, and provides unique insight into the molecular regulators of satellite cell senescence during ageing. Further the evaluation of miRs as molecular regulators of redox biology is an emerging area, of interest to a wide readership.

Thank you for this comment, we agree that the manuscript presents novel data relevant to both microRNA and ageing fields.

This manuscript would likely garner attention from miRNA researchers outside of the muscle field. Thus, I feel the BaCl2 experiments needs a little more information and perhaps justification, to provide context in the type and magnitude of injury induced by this model and its applicability to studying muscle ageing. If the authors have any tangible data (perhaps included in the supplementary figures), then I think this could be a useful addition.

Thank you for this suggestion. Local injections with Barium Chloride ($BaCl_2$) is a widely used model of skeletal muscle injury, established decades ago for the study of muscle generation and repair *in vivo* (Cerri et al. 2008, Kim et al. 2016). This model has been used by many researchers since it relatively inexpensive but an elegant approach, also comparable to other conventional acute injury models such as cardiotoxin (Hardy et al. 2016, Jung et al. 2019). Particularly, local muscle injury with $BaCl_2$ has been shown to induce depolarization of the sarcolemma, membrane rupture, proteolysis and motor denervation in the skeletal muscle fibers (Morton et al. 2019). However, BaCl2- induced muscle damage preserves satellite cells allowing for detailed study of their role in muscle regeneration (Morton et al., 2019). Through a straightforward intramuscular injection, a significant proportion of muscle is damaged and the regeneration follows through infiltration of immune cells, satellite cells activation with most of the damage resolved by 21 days in adult mice. However, in old mice, this damage is not resolved within 21 days, therefore this model is considered appropriate for studying changes in muscle regeneration during ageing (Fig. 1K). Representative images have been now included in the revised manuscript in Fig. 1K.

References:

Cerri, D. G., L. C. Rodrigues, S. R. Stowell, D. D. Araujo, M. C. Coelho, S. R. Oliveira, J. C. S. Bizario, R. D. Cummings, M. Dias-Baruffi and M. C. R. Costa (2008). "Degeneration of dystrophic or injured skeletal muscles induces high expression of Galectin-1." Glycobiology **18**(11): 842-850.

Hardy, D., A. Besnard, M. Latil, G. Jouvion, D. Briand, C. Thépenier, Q. Pascal, A. Guguin, B. Gayraud-Morel, J.-M. Cavaillon, S. Tajbakhsh, P. Rocheteau and F. Chrétien (2016). "Comparative Study of Injury Models for Studying Muscle Regeneration in Mice." PLOS ONE **11**(1): e0147198.

Jung, H.-W., J.-H. Choi, T. Jo, H. Shin and J. M. Suh (2019). "Systemic and Local Phenotypes of Barium Chloride Induced Skeletal Muscle Injury in Mice." Annals of geriatric medicine and research **23**(2): 83-89.

Kim, J. H., G. C. Han, J. Y. Seo, I. Park, W. Park, H. W. Jeong, S. H. Lee, S. H. Bae, J. Seong, M. K. Yum, S. H. Hann, Y. G. Kwon, D. Seo, M. H. Choi and Y. Y. Kong (2016). "Sex hormones establish a reserve pool of adult muscle stem cells." Nat Cell Biol **18**(9): 930-940.

Morton, A. B., C. E. Norton, N. L. Jacobsen, C. A. Fernando, D. D. W. Cornelison and S. S. Segal (2019). "Barium chloride injures myofibers through calcium-induced proteolysis with fragmentation of motor nerves and microvessels." Skelet Muscle **9**(1): 27.

I was particularly interested in the mooted role of modified ROS generation in this model; however, I am not clear on the rationale of the experimental design – in the context of spiking the samples with hydrogen peroxide. Given the modified satellite cell function observed during ageing, I would have expected the authors to measure ROS using the typical family of fluorescent probes. Could the authors please clarify further (or more explicitly) why they chose to introduce hydrogen peroxide (mimicking oxidative stress), rather than measure endogenous ROS generation in their model, in the absence of H2O2?

The reviewer has made a good point, DCFH-DA is quite unspecific in terms of a measure of overall ROS and some of the chemistry is not fully understood. As mentioned we have repeated all previous experiments using primary mouse (young and old) and human myoblasts treated with miR-24 or antiiR-24 but have also included a group treated with siPrdx6 in the revised manuscript. Since Prdx6 has been previously shown to regulate mitochondrial ROS generation, we have focused on the measurement of MitoSox Red as an indicator of mitochondrial generated superoxide (Figs. 3F,H, 6C,F). In addition, we stained the mitochondria with Mitotracker Red to visualise their morphology (Figs. 3E, 6C).

Following on from my prior point, do the author have any gauge on whether markers of oxidative damage follow the trend they observed in CA-DCFH-DA fluorescence?

This is an excellent suggestion by the reviewer and we have now included a quantification of markers of DNA damage (H2AX) to compliment the increase in Mitosox in miR-24 and siPrdx6 treated cells, coupled with the increase in senescent cells. We have also included in the revised manuscript images of mitochondrial networks in these cells visualised using MitoTracker Red and have included Western blots analyses of the expression of Prdx6 and Prdx3 in young and old mouse primary myoblasts and human primary myoblasts (Fig3.E-H and Fig4.F-G). We found that consistent with the original data and Prdx6 downregulation resulted in altered morphology of mitochondrial networks, increased mitochondrial ROS generation and increase in DNA damage marker, however this was not associated with increase in PRDX3 levels (Figs.3,6). Interestingly, inhibition of miR-24 and downregulation of Prdx6 had an effect on HSP70 levels, which was different between cells from adult and old mice, which may provide further clarification on the more pronounced phenotype elucidated by miR-24 in cells from old mice (Fig. 4F,G).

Further, can the authors clarify the use of the DCFH-DA probe over other fluorescent probes? I am aware this is mentioned as a limitation; however, I feel the choice needs justification. I take a very pragmatic view on this, since there is not necessarily always an adequate choice, with many of the tools we used to measure ROS indirectly, have their own limitations.

The reviewer has made an excellent point and we agree that the original experiment in which cells were treated with H2O2, likely masking the effect of miR-24 and Prdx6, as well as using an unspecific ROS probe, was not very informative. Therefore, as mentioned previously in the revised manuscript where we have repeated all work on mouse and human primary myoblasts to include the siPrdx6 group, we have used Mitosox as an indicator of mitochondrial superoxide generation (Figs. 3F,H, 6C,F).

In some instances, modulation of specific antioxidant defence enzymes can invoke compensatory changes in others. Do the authors have any insight as to whether siRNA suppression of Prdx6 induced any compensatory responses in other systems.

Again this is an excellent point and we have analysed the expression of Prdx3, Hsp70 and SOD1 along with Prdx6, key antioxidant and chaperone proteins within the cell. On the protein levels, we consistently detected downregulation of PRDX6 in myogenic progenitors from adult and old mice, however the variability between the experimental groups was high and this is reflected throughout manuscript. Nevertheless, we are confident with the data as the phenotype elucidated by miR-24 and siPrdx6 was consistent between different assays used and between experiments performed in the original and revised manuscripts, both performed using different sets of reagents (due to discontinuation of miR-24 antimiR, control RNA and siRNA Prdx6 used in the original manuscript). We detected significantly decreased expression of Prdx6 in human primary myoblasts (Fig.6G) and adult myoblast treated with siPrdx6 but this did not reach significance in old primary myoblasts. This was associated with no change in PRDX3 or SOD1 levels and an increase in the expression of the chaperone protein HSP70 in primary myoblasts from adult but not old mice (Fig.4F,G, S6). These data suggest a potential mechanism by which miR-24:Prdx6 interactions may regulate cell senescence more robustly in the cells from old mice.

The authors use siRNA to supress Prdx6 expression in several experiments, could you please provide some information on the knockdown efficiency of your models?

The efficiency of the siPrdx6 was much more effective in human primary myoblasts with a very clear decrease in protein expression (~25%) (Fig. 6G). The change in expression of Prdx6 following siRNA in primary myoblasts from mice was ~50%, that reached significance in young but not old cells. We have repeated these experiments multiple times and the variability in the efficiency of transfections is quite high using siRNA as compared to miR mimic or antimiRs (Figs. 4. D,E,F,G, S2).

Similarly, could you provide some quantitative measure of transfection efficiency across all experiments?

In addition to the transfection efficiency observed when using siPrdx6 above, for 100nM miR mimic and antimiR, we observe >50% transfection efficiency in myoblasts as determined by fluorescently-labelled control mimics. However, we optimise the concentration of miR mimic or antagomiR based on significant up- or downregulation of miR, respectively. We aim for 2-fold increase or reduction in expression. These data are shown in Fig.S2.

Reviewer 2

In this paper the authors identify miR-24 as a potential regulator of factors involved in oxidative stress in satellite cells. The use a range of studies to examine the expression of miR-24 in muscle and in sorted muscle satellite cells from old and young mice. They identify miR-24 as differentially expressed between satellite cells from old and young mice. They examine the effects of both the miRNA and its antagomiR (AM24) on cell viability and on myotube formation. In old cells miR-24 reduces senescence and in adult cells AM24 reduces senescence. The gene expression data presented alongside are a little difficult to interpret as there is an increase in senescence markers in the adult cells in response to miR-24 but a reduction in old cells. Whether this is a consequence of the relative proportions of already senescing cells in the two different populations is difficult to know.
The they show miR-24 can target Prdx6 and they examine the ability of miR-24 and AM24 to regulate Prdx6 mRNA and ROS. Finally they look at the effects of the miR and antagomiR in human cells on the same parameters. Overall, I find the data a little confusing as there are different (and sometimes opposing) effects of miR-24 and AM24 dependent on the age of the cells. If this is the case it is essential to pin down the real reasons and I am not sure that they manage this in the paper as presented. In particular they try to reason that Prdx6 is the key and I am not convinced that they succeed (see major point 3).

We would like to thank the reviewer for their comments and agree with the reviewer that the results were not always clear in the original submitted manuscript. We have repeated all the cell work using primary myoblasts from humans and mice to include a group with siPrdx6. We believe the data presented in the revised manuscript is a lot cleaner and supports our previous results on the role of Prdx6 in senescent cells.

Specifically, we further explored the role of miR-24 on senescence and optimised our experiment. As discussed in the manuscript, page 9:

According to published data, miR-24 regulates the expression of tumour suppressor/senescence-associated proteins differently depending on the cell type and metabolic state of the cell: it reduced P16 protein levels in human diploid fibroblasts and cervical carcinoma cells and it inhibited H2Ax in terminally differentiated hematopoietic cells making them vulnerable to DNA damage (Lal et al., 2009). On the other hand, miR-24 has been shown to increase p53 and p21 protein levels in different cancer cell lines (Mishra et al., 2009); and to induce p53 expression in human epithelial cells during aging and oxidative stress (Lu et al., 2018). Together, these results suggest that miR-24 exerts either an inhibitory or enhancer function over tumour suppressor/senescence-associated proteins depending on the cell cycle state, which is consistent with our findings. Context-dependent role of miRs has been previously demonstrated, as well as their dose-dependent regulation of physiological processes (Vasudevan, 2012).

As mentioned above, miR-24 targets several genes associated with cellular senescence and we agree with the reviewer that Prdx6 is, although important, is one of many physiologically relevant miR-24 targets. However, the date consistently indicate similarities between miR-24 overexpression and Prdx6 downregulation phenotypes. We agree that AM24 had limited effect on cellular senescence. This may be because the cells entered an irreversible senescence or the effect was not strong enough to be detected (Figs. 3A, 6A). Nevertheless, AM24 clearly promoted cell survival and myogenic differentiation (Figs. 2, 5).
miR-24 may have a different role on regulating cell cycle-associated genes depending on their cell cycle status as indicated by the acute increase in expression following muscle injury in adult mice (Fig. 1J). In the original manuscript, we used late passage cells with populations of approximately

50% cells showing replicative senescence. Moreover, the cells used were from a wide range of mice (adult 1-8 months old and old 20-24 months old). As dynamic changes occur in muscle during these ages, we optimised our approach in the revised manuscript and used cells at P4-P7 for all assays and cells from 6 (adult) or 24 (old) months old mice. Whilst miR-24 had a stronger effect on cells from old mice, the phenotypic effects were the same between cells from adult and old mice: miR-24 upregulation, as well as downregulation of its target Prdx6, was in all cases associated with an increase in cell senescence, as well as increased ROS generation and increase in the marker of DNA damage.

Moreover, we have explored further how miR24:Prdx6 may regulate cell viability, myogenic potential and senescence during ageing. Through exploring mitochondrial network morphology and ROS generation, as well as maker of DNA damage and changes in the levels of senescence (p16, p53). We believe that the data support the role of miR-24:Prdx6 in regulating viability and senescence pathways through increase in ROS generation and DNA damage which is associated with changes in viability and senescence upon miR-24 upregulation and downregulation of Prdx3. This is now discussed in the revised manuscript.

The authors claim in that the number of satellite cells goes down, the figure says % these are not necessarily the same thing as there could be an increase in fibroblasts or inflammatory cells in the old for the same number of satellite cells and that would give the same result.

Raw numbers would not provide a realistic interpretation of the data since the starting material (amount of muscle collected), as well as technical variabilities during the different FACS performed (such as longer sorting times), will affect the total number of cells sorted. We show in our FACS results that the % of satellite cells over the total number of cells decreases in the old mice compared to the adult mice. However, the results demonstrated in this manuscript are consistent with previously published data on decrease in satellite cell number during ageing (e.g. Snow et al, The effcts of aging on satellite cells in skeletal msucles of mice and rats; Cell Tissue Res, 1977; Verdijk et al., Reduced satellite cell numbers with spinal cord injury and aging in humans; Med Sci Sports Exerc, 2012).

Normalisation of data and use of t-Tests. I am a little concerned about the use of t-tests for data that has been normalised to 1 to allow the comparison of replicate experiments. Whilst it is common practice, I am not sure that it is correct. The t-test requires a normal distribution and this approach normally violates it in 2 ways. Firstly, the value 1 has no distribution so cannot be said to have a normal distribution and secondly the ratios are often not normally distributed because a two-fold reduction produces 0.5 whereas a two-fold increase is 2. If you use this approach to random data it is easy to generate a statistically significant increase with just noise. I realise that many of the differences observed are decreases but the point still stands. There needs to be a variance on the normalised value for a t-test to be applicable.

We agree with the reviewer. We originally normalised the data as described above, the variability of the control cell phenotype was high, most likely due to the wide range of ages of mice used to isolate myogenic progenitors. In the revised manuscript, all data collection has been repeated using 6- and 24-month mice and cells.

All statistical analyses have been performed on raw values; this information is contained within each figure legend. T test have only been used for pairwise comparison if data was deemed normally distributed, otherwise Mann Whitney test was used. For multiple comparisons, we used One Way A.N.O.V.A followed by Tukey's multiple comparison or Kruskal Wallis test followed by Dunn's multiple comparison, as indicated in the figures.

We are happy to note that between the two different experimental settings and two sets of different reagents: miR-24, AM24 and siPrdx6, all data is consistent in terms of the phenotype

induced by miR-24 and Prdx6. We included original data in the supplementary figures for the purpose of transparency and further support of the conclusions.

I am not sure that the miR-AM24 data really support the suggestion that the miRNA targets Prdx6 to increase oxidative stress. The data in Fig 4 show that there is more PrdX6 in cells from old mice(A), that there is a potential target site in the 3'-UTR (B) and that this site is targeted by miR-24 (C). However, any suppression of Prdx6 mRNA by additional miR-24 is small and only seen in old mice and there is no real effect of the AM24 (D/E). There is no western blot data to show reduced protein (which may have a larger effect).

We have analysed the expression of Prdx3, Hsp70 and SOD1 along with Prdx6, key antioxidant and chaperone proteins within the cell. On the protein levels, we consistently detected downregulation of PRDX6 in myogenic progenitors from adult and old mice, however the variability between the experimental groups was high and this is reflected throughout manuscript. Nevertheless, we are confident with the data as the phenotype elucidated by miR-24 was consistent between different assays used and between experiments performed in the original and revised manuscripts, both performed using different sets of reagents (due to discontinuation of miR-24 antimiR, control RNA and siRNA Prdx6 used in the original manuscript).
We detected a significant decreased expression of Prdx6 in human primary myoblasts (Figs. 4F,G, 6G) and adult myoblast treated with siPrdx6 but this did not reach significance in old primary myoblasts. This was associated with no change in PRDX3 or SOD1 levels and an increase in the expression of the chaperone protein HSP70 in primary myoblasts from adult but not old mice (Figs. 4F,G, 6G, S6). These data suggest a potential mechanism by which miR-24:Prdx6 interactions may regulate cell senescence more robustly in the cells from old mice.

The data in F, G and H are suggested to show changes in ROS production in the presence of H2O2. However, the size of the difference in the oxidative stress markers following transfection seems very small. Given the data are normalised to 1 the increase is 2.5%. The images also show a much larger apparent difference but the difference is restricted to what look like cells in clumps so it would be useful to see brightfield images to accompany the fluorescent images. Finally, I am not sure that the data in G and H really support the conclusion that is drawn.
In young mice there are no significant differences, whereas there are some differences in the old mice. The argument given is that there is more miR-24 in the young mice therefore less Prdx6 (Figs 1H and 4A). Consequently, if miR-24 was a major contributor to the reduction in Prdx6 then the AM24 should have a larger effect in the young mice than in the old but this doesn't happen, indeed there is no effect of the AM24 in the young mice. Similarly, as the amount of ROS precursor (H2O2) added is the same and there should be more Prdx6 in the old cells then if Prdx6 was the major regulator of oxidative stress in the system then there should be greater ROS production in the young mouse samples (due to less Prdx6) than in the old mouse samples but from the images this isn't the case.
Furthermore, the significant differences for miR-24 in the old mice are between transfection with miR-24 and transfection with the AM24 and not between either and the control. The argument here is that the levels of miR-24 are low so there is no effect of the AM24. However, as the levels of Prdx6 are very high there ought to be a significant effect of the miR and there isn't.
Furthermore, knockdown of Prdx6 increases ROS (siRNA data) but this increase is suppressed by addition of the AM24. As Prdx6 has been knocked down by an siRNA how can the effects of the AM24 on ROS be via Prdx6 as there is no Prdx6 for miR-24 to be suppressing so no effect for the AM24 to reverse. Any effect of the AM24/miR would have to be by targeting a separate component of the ROS pathway or an indirect effect on Prdx6 expression (though this seems to be ruled out by Fig 4D/E). This later argument also applies to the data in Fig 6B where again the AM24 reverses the effect of the siRNA.

As reviewer 1 commented, DCFH-DA is quite unspecific in terms of a measure of overall ROS and some of the chemistry is not fully understood. As mentioned above, we have repeated all previous experiments using primary mouse (young and old) and human myoblasts treated with miR-24 or antagomiR-24 but have also included a group treated with siPrdx6 in the revised manuscript. Since Prdx6 has been previously shown to regulate mitochondrial ROS generation, we have focused on the measurement of MitoSox Red as an indicator of mitochondrial generated superoxide. In addition, we stained the mitochondria with Mitotracker Red to visualise their morphology (Figs. 3E,F,H, 6C,D,F). We did not treat the cells with H2O2 as indicated in Figure S5, treatment of H2O2 produces a massive induction of ROS generation, which was likely masking the effects of miR-24 and Prdx6 in the original manuscript.

We believe that the more pronounced effects of miR-24 in cells from old mice are the result of miR-24 regulating other genes, such as p21, and not due to different levels of Prdx6 between adult and old cells (please see page 9 discussion and above) – we would like to thank the reviewer for pointing this out.

Reviewer is correct and there are multiple possible pathways through which Prdx6 can exert its effects, as Prdx6 has a number of known functions as a peroxidase and phospholipase activities, indeed the latter of which can potentially promote ROS production via its role in the activation of Nox2. The data presented in the revised manuscript would confirm our original hypothesis that Prdx6 has a key role in senescence via the increase in ROS generation and overall DNA damage. Nevertheless, we acknowledge this limitation of our data in the revised manuscript (page 10):
Moreover, the diverse activities of Prdx6, including peroxidase, PLA2 phospholipase and LPCAT activities, mean that it could potentially regulate different metabolic signalling pathways, from cell cycle, membrane repair and antioxidant response (Arevalo & Vázquez-Medina, 2018; López Grueso et al., 2019). The limitation of this manuscript is that it did not explore the function of Prdx6 as PLA2 phospholipase in the context of muscle ageing.

Line 177: position 2-6 (6mer): should be 5mer

Thank you, this has been corrected.

AGING CELL AUTHOR CHECKLIST. *Authors should submit this checklist together with their manuscript. Please ensure that you have read the Author Guidelines in detail before submission.*

Title	miR-24 and its target gene Prdx6 regulate viability and senescence of myogenic progenitors during aging
Authors	Ana Soriano-Arroquia, John Gostage, Qin Xia, David Bardell, Rachel McCormick, Eugene McCloskey, Ilaria Bellantuono, Peter Clegg, Brian McDonagh, Katarzyna Goljanek-Whysall
Manuscript Type	Research Report
Total Character Count (including spaces)[1]	47,158
Word count of Summary[2]	247
Number of papers cited in the References[3]	45
Listing of all Tables (Table1, Table 2 etc)[4]	Table S1, Table S2

Figure specifications (please complete one row per figure)[5] *Figure no.*	Colour (yes/no)	Greyscale (yes/no)	Black and white (yes/no)	Single column (80mm) (yes/no)	Double column (167mm) (yes/no)	Size of figure at full scale (mm x mm) *(insert details)*	Smallest font size used in the figure at full scale (minimum 6pt) *(insert details)*
1	Yes	No	No	No	Yes	160x204	6pt
2	Yes	No	No	No	yes	150x175	6pt
3	Yes	No	No	No	yes	160x228	6pt
4	Yes	No	No	No	Yes	160x165	6pt
5	Yes	No	No	No	Yes	152x91	6pt
6	Yes	No	No	No	Yes	137x112	6pt
Supplementary 1	Yes	No	No	No	Yes	194.5x162.7	6pt
Supplementary 2	Yes	No	No	No	Yes		6pt

Supplementary 3	Yes	No	No	No	Yes	160x131	6pt
Supplementary 4	Yes	No	No	Yes	No	160x150	6pt
Supplementary 5	Yes	No	No	Yes	No	76x124	6pt
Supplementary 6	Yes	No	No	Yes	No	94x120	6pt
Graphical abstract	Yes	No	No	Yes	No	50x60	6pt

[1] The maximum character count allowed is 50,000 (incl. spaces) for Primary Research Papers, 10,000 for Short Takes and 20,000 for Reviews.

[2] Summary should not exceed 250 words.

[3] Primary Research Papers can contain a maximum of two tables. If more are needed they should replace some of the Figures or can be placed in the Supporting Information.

[4] A maximum of 45 references is allowed for Primary Research Papers and 20 references for Short Takes.

[5] A Primary Research Paper may contain up to 6 figures and a Short Take up to 2 figures. Authors are encouraged to provide figures in the size they are to appear in the journal and at the specifications given.

miR-24 and its target gene Prdx6 regulate viability and senescence of myogenic progenitors during aging

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34

GRAPHICAL ABSTRACT

Age-related changes in miR-24 and its target gene Prdx6 contribute to defective function of myogenic progenitors and muscle regeneration during aging. miR-24 upregulation and downregulation of its target Prdx6 is associated with an increase in mitochondrial ROS, increase in pH2Ax, decreased cell viability, myogenic potential and increased senescence. During aging, downregulation of miR-24 in satellite cells and after muscle injury may represent a compensatory mechanism acting to preserve cell viability and myogenic potential.

ABSTRACT

Satellite cell-dependent skeletal muscle regeneration declines during aging. Disruptions within the satellite cells and their niche, together with alterations in the myofibrillar environment contribute to age-related dysfunction and defective muscle regeneration.

In this study, we demonstrated an age-related decline in satellite cell viability and myogenic potential, and an increase in ROS and cellular senescence. We detected a transient upregulation of miR-24 in regenerating muscle from adult mice and downregulation of miR-24 during muscle regeneration in old mice. FACS-sorted satellite cells were characterised by decreased levels of miR-24 and a concomitant increase in expression of its target: Prdx6. Using GFP reporter constructs, we demonstrated that miR-24 directly binds to its predicted site within Prdx6 mRNA. Subtle changes in Prdx6 levels following changes in miR-24 expression indicate miR-24 plays a role in fine-tuning Prdx6 expression. Changes in miR-24 and Prdx6 levels were associated with altered mitochondrial ROS generation, increase in the DNA damage marker: phosphorylated-H2Ax and changes in viability, senescence and myogenic potential of myogenic progenitors from mice and humans. The effects of miR-24 were more pronounced in myogenic progenitors from old mice, suggesting a context-dependent role of miR-24 in these cells, with miR-24 downregulation likely a part of a compensatory response to decline in cell function during aging.

We propose that downregulation of miR-24 and subsequent upregulation of Prdx6 in muscle of old mice following injury is an adaptive response to aging, to maintain satellite cell viability and myogenic potential through regulation of mitochondrial ROS and DNA damage pathways.

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68 **INTRODUCTION**

69 The regenerative capacity of skeletal muscle facilitates a high plasticity for adaptation to diverse

70 metabolic conditions and energetic demands. Skeletal muscle regeneration after injury and loading

71 stressors relies on satellite cells, the adult muscle stem cells able to regenerate muscle fibres *in vivo*.

72 A balance between satellite cells self-renewal and myogenic differentiation is essential for successful

73 muscle regeneration after injury (Sambasivan & Tajbakhsh 2015). However, the effectiveness of

74 muscle regeneration throughout lifespan not only relies on the functionality of satellite cells (Lepper

75 *et al.* 2011), ~~but also other factors, such as~~ disrupted intracellular signalling and an altered muscle

76 fibre microenvironment are known to play a key role in muscle wasting during disuse, ageing and

77 chronic diseases (Fry *et al.* 2015; Le Moal *et al.* 2017). In particular, oxidative stress has been

78 demonstrated to alter the cellular microenvironment, resulting in disrupted cellular signalling and

79 potentially oxidative modifications of muscle contractile proteins (Goljanek-Whysall *et al.* 2016;

80 Sakellariou *et al.* 2017). Some of the important muscle antioxidant proteins that directly affect

81 intracellular ROS concentrations are members of the peroxiredoxin family: (PRDX1-PRDX6).

82 Peroxiredoxins have the capacity to regulate redox homeostasis and signalling pathways involved in

83 processes such as apoptosis and cell survival or in response to injury. Particularly, Peroxiredoxin 6

84 (Prdx6) has been demonstrated to regulate both myogenesis and adipogenesis *via* the control of

85 glucose uptake (Pacifici *et al.* 2014; Wu *et al.* 2015), and *Prdx6$^{-/-}$* mice display increased levels of

86 markers of senescence, metabolic sarcopenia and loss of muscle strength (Pacifici *et al.* 2020).

87

88 microRNAs (miRNAs, miRs) are short non-coding RNAs approximately 20-22 nucleotides in length.

89 miRs show partial complementarity to their target mRNA(s) and regulate gene expression at the post-

90 transcriptional level (Lee *et al.* 1993). miRs are known to regulate a myriad of biological processes,

91 including muscle homeostasis and aging through processes such as ROS generation and scavenging

92 (Goljanek-Whysall *et al.* 2020). miR-24 is highly expressed in skeletal muscle (Wada *et al.* 2011) and

93 has been proposed to regulate myogenesis *in vitro* and to inhibit muscle fibrosis *in vivo* (Sun *et al.*

94 2008; Sun *et al.* 2018). Yet, the functional role of miR-24 in human and mouse primary myogenic

95 stem/progenitor cells, including oxidative stress, and in skeletal muscle aging remains elusive.

96

97 In this study, we identified changes in miR-24:Prdx6 interactions in satellite cells during aging. Our

98 data confirm a decline in satellite cell fraction, viability and myogenic potential in muscle from old

99 mice. miR-24 expression was downregulated in FACS-sorted satellite cells during aging, with the

100 concomitant upregulation of its target Prdx6. Our results demonstrate a transient upregulation of miR-

101 24 in regenerating muscle from adult mice after acute injury, whereas in old mice, we detected

102   downregulation of miR-24 expression during muscle regeneration. Using GFP reporter constructs, we

103   demonstrated the binding of miR-24 to its target site within Prdx6 mRNA. Changes in miR-24:Prdx6

104   interactions were associated with altered mitochondrial ROS generation and levels of phosphorylated-

105   H2Ax in myogenic progenitors and affected their viability, myogenic potential and senescence. The

106   effects of miR-24 up- and downregulation were more pronounced in myogenic progenitors from old

107   mice, suggesting a context-dependent role of miR-24 in these cells. We propose that changes in miR-

108   24:Prdx6 interactions during aging are aimed at preserving satellite cells viability and function. We

109   hypothesise that age-related downregulation of miR-24 and subsequent increased Prdx6 expression

110   in satellite cells represents an adaptive mechanism aimed to improve the regenerative capacity of

111   skeletal muscle through preserving satellite cell viability and function by regulating ROS-associated

112   pathways.

113

114   **RESULTS**

115   **miR-24 is downregulated during skeletal muscle regeneration and aging**

116   miR-24 has been previously shown to be regulated during satellite cell activation (Cheung *et al.* 2012;

117   Redshaw *et al.* 2014). Some of the miR-24 putative targets in humans, analysed using TargetScan and

118   ClueGO plugin for Cytoscape, were genes associated with the cellular response to oxidative stress,

119   including Prdx6 **(Figure 1A)**. We focused on miR-24 target genes associated regulation of viability,

120   differentiation and senescence through regulation of redox balance, as redox homeostasis has been

121   shown to regulate all these processes during aging (Le Moal *et al.* 2017). Prdx6 has been shown to

122   regulate skeletal muscle adaptation under increased oxidative stress (Da Silva-Azevedo *et al.* 2009).

123   Prdx6 was upregulated in FACS-sorted satellite cells isolated from old mice compared to adult mice

124   **(Figure 4A)**, but not in the tibialis anterior muscle from old mice **(Figure S2).** This is consistent with

125   downregulation of miR-24 expression in satellite cells but not muscle during aging **(Figure 1H, J)**. We

126   therefore investigated age-related changes in miR-24:Prdx6 interactions in satellite cells. We observed

127   a decrease in the total number of FACS-sorted satellite cells during aging **(Figures 1B, S1**), consistent

128   with previously published data **(Gibson & Schultz 1983).** Myogenic progenitors from old mice

129   displayed increased senescence **(Figures 1C, 3A)**, reduced viability **(Figures 1D, 2A)**, reduced myogenic

130   potential **(Figures 1E, 2D)** and increased ROS **(Figures 1F, 1G)**. miR-24 expression was downregulated

131   in satellite cells from old mice **(Figure 1H)**. The expression of miR-24 was also analysed by RT-qPCR in

132   an *in vivo* model of skeletal muscle regeneration following barium chloride (BaCl$_2$) injection **(Figures

133   1I, 1J, 1K)**. Local muscle injury with BaCl$_2$ has been shown to induce depolarization of the sarcolemma,

134   membrane rupture, proteolysis and motor denervation in the skeletal muscle fibers (Morton et al.

135   2019). However, BaCl2- induced muscle damage preserves satellite cells allowing for detailed study of

136   their role in muscle regeneration (Morton et al., 2019). Most of the damage resolved by 21 days in

137   adult but not old mice, in which central nuclei remain after 21 days (**Figure 1K**). miR-24 basal levels

138   were not altered in TA muscle during aging, as opposed to its downregulation in satellite cells **(Figure**

139   **1H, 1J)**, but its expression was increased one day after muscle injury and returned to basal levels after

140   seven days in the injured muscle of adult mice, suggesting a potential role of miR-24 in the early stages

141   of muscle regeneration after acute injury. However, the expression of miR-24 did not increase in old

142   mice following injury: miR-24 expression was significantly lower at 1-21 days after injury compared to

143   the adult mice **(Figure 1J)**. These data suggest that the downregulation of miR-24 in satellite cells

144   **(Figure 1H)** may be related to the age-related decline in satellite function and consequently muscle

145   regeneration following acute injury.

146   **miR-24 regulates viability and myogenic potential of myogenic progenitors during aging**

147   The function of satellite cells in muscle regeneration depends on their viability and myogenic

148   potential, both are affected by aging **(Figures 1D, 1E)**. To determine the physiological consequences

149   of miR-24 and its target Prdx6 dysregulation in satellite cells during aging and regeneration, myogenic

150   progenitors isolated from adult and old mice were transfected with miR-24 mimic, AM24, siRNA for

151   Prdx6 or scrambled RNA (control) and stained to evaluate differentiation (MF 20), proliferation (Ki67)

152   and viability **(Figures 2, S4)**. miR-24 had no significant effect on myogenic progenitor proliferation

153   (**Figure S3**). However, overexpression of miR-24 and downregulation of Prdx6 expression resulted in

154   the increased proportion of necrotic and apoptotic myogenic progenitors from adult and old mice and

155   inhibition of miR-24 resulted in an increase in the total number and number of viable myogenic

156   progenitors **(Figure 2A, B)**. Moreover, overexpression of miR-24 and downregulation of its target:

157   Prdx6, in myogenic progenitors from adult and old mice resulted in inhibition of myogenesis **(Figure**

158   **2C, D)**. These data were consistent with previous, independently performed analyses of miR-24 role

159   in myogenic progenitors **(Figure S3)**.

160   **miR-24 and its target Prdx6 regulate senescence of myogenic progenitors.**

161   Satellite cells have been previously shown to undergo senescence during aging (Blau *et al.* 2015; Zhu

162   *et al.* 2019). Adult and old myogenic progenitors at passage 7, Senescent cells become present in both

163   culture from adult and old myogenic progenitors from at passage 7, cells were then transfected with

164   miR-24 mimic or inhibitor (AM24) or Prdx6 siRNA. Overexpression of miR-24 or Prdx6 downregulation

165   led to a higher proportion of senescent cells in myogenic progenitors from both adult and old mice,

166   as well as higher number of senescent cells as measured by SA-βgal staining and by measurement of

167   fluorescent SA-βgal. **(Figure 3A-C)**. The expression of senescence-associated genes p16 and p53 was

168   not changed in myogenic progenitors from adult mice following miR-24 overexpression or Prdx6

169   downregulation **(Figure 3D)**. In myogenic progenitors from old mice, miR-24 overexpression and

170   downregulation of Prdx6 led to increased p16 and p53 levels, which were significantly different in cells

171   treated with siRNA for Prdx6 (**Figure 3D**). However, inhibition of miR-24 in myogenic progenitors from

172   old mice had no significant effect on number of SA-βgal positive cells or the overall proportion of

173   senescent cells or expression of senescence-associated genes **(Figure 3A-D)**. This could be associated

174   with already low levels of miR-24 in cells from older mice or altered levels of other target genes of

175   miR-24, such as p21 in myogenic progenitors from adult and old mice (Lal et al., 2009)(Mishra et al.,

176   2009)(Lu et al., 2018). The latter is supported by our initial assessment of the role of miR-24 in

177   regulating senescence of myogenic progenitors, where miR-24 had different effects on changes in

178   senescence-associated gene expression in senescent cells from adult and old mice, despite similar

179   effects on cellular senescence on the phenotypic level (**Figure S3C,E**).

miR-24 and its target Prdx6 regulate mitochondrial ROS production and the levels of DNA damage

marker.

182   As Prdx6 has been previously shown to regulate mitochondrial dynamics and function, myogenic

183   progenitors cells transfected with miR-24, AM24 or Prdx6 siRNA and stained with MitoTracker Red to

184   visualise mitochondria (Figure 3E), as well as Mitosox Red and phosphorylated H2Ax, to detect

185   mitochondrial ROS and DNA damage, respectively (**Figure 3E-H**). Myogenic progenitors from both

186   adult and old mice showed disrupted mitochondrial morphology, increased mitochondrial ROS

187   production and increase in DNA damage marker following overexpression of miR-24 or

188   downregulation of Prdx6 expression (**Figure 3E-H**). These effects were more pronounced in myogenic

189   progenitors from old mice, consistent with the differences in regulation of senescence by miR-24 in

190   myogenic progenitors from adult and old mice, suggesting a context-dependent function of miR-24.

191   Together, these data indicate a potential mechanism of regulation of myogenic progenitor senescence

192   and viability by miR-24 and its target Prdx6, where increased levels of miR-24 and concomitant

193   downregulation of Prdx6 lead to disruption of mitochondrial morphology, increase in mitochondrial

194   ROS production, increase in DNA damage marker levels and induction of pro-apoptotic and/or pro-

195   senescent pathways, likely through p16 and p53 signalling.

miR-24 directly regulates the expression of Prdx6 in myogenic progenitors

197   Prdx6 is a confirmed miR-24 target in human cells (Li *et al.* 2016). We next analysed the sequence of

198   mouse Prdx6 for miR-24 binding sites. A target site for miR-24 was found between position 2-6 (5-

199   mer) of the mature microRNA-24 and position 41-45 5'UTR of the mouse Prdx6-202 transcript **(Figure**

200   **4B)**. A GFP reporter containing the wild type or mutated miR-24 binding site for miR-24 was generated

201   **(Figure 4C)**. C2C12 myoblasts were transfected with reporter constructs containing wild type or

202   mutated miR-24 binding site within the Prdx6 5'UTR fragment and co-transfected with miR-24 mimic

203   or scrambled sequence (control). GFP levels were decreased in the cells transfected with wild type

204   construct co-transfected with miR-24 as compared to scrambled treated cells, but not in the cells

205   treated with the mutated construct co-transfected with miR-24 or control Scr microRNA These results

206   confirm that miR-24 directly binds to Prdx6 mRNA in mouse myoblasts **(Figure 4C)**. We next

207   investigated Prdx6 expression following miR-24 overexpression or downregulation in myogenic

208   progenitors from adult and old mice. Prdx6 expression was significantly downregulated following miR-

209   24 overexpression in myogenic progenitors from old but not adult mice **(Figure 4E)**. Similarly, protein

210   levels of PRDX6 were affected by miR-24 levels in old but not adult mice, although these changes did

211   not reach statistical significance, likely due to relatively small n number (n=3). Cell treated with Prdx6

212   siRNA showed lower levels of PRDX6, although the level of downregulation varied between individual

213   replicates **(Figure 3F,G)**. Interestingly, changes in the levels of miR-24 or Prdx6 did affect the levels of

214   a mitochondrial peroxiredoxin PRDX3. However, inhibition of miR-24 or downregulation of Prdx6

215   levels resulted in changes in the levels of chaperone protein HSP70 in adult progenitors suggesting a

216   potential mechanism underlying the phenotypic differences between the effects of miR-24 on

217   myogenic progenitors from adult and old mice **(Figure 4F,G)**.

218   **miR-24 regulation of myogenic potential and viability by controlling Prdx6 is conserved in human**

219   **myogenic progenitors**

220   We further explored whether the function of miR-24 and its target Prdx6 is conserved in human cells.

221   Myogenic progenitors isolated from adults were transfected with miR-24 mimic, AM24, siRNA for

222   Prdx6 or scrambled control. miR-24 overexpression and downregulation of Prdx6 expression resulted

223   in decreased cell viability and lower total cell number as well as decreased myogenic potential with

224   miR-24 overexpression and siPrdx6 downregulation resulting in the presence of smaller myotubes

225   containing fewer nuclei **(Figure 5A-D)**. miR-24 had no effect on proliferation of human myogenic

226   progenitors **(Figure S4)**, consistently with the lack of miR-24 regulation of murine cell proliferation.

227   These data are consistent with murine data and our initial assessment of miR-24 role in human

228   myogenic progenitors **(Fig.S4 A,B)**.

229

230   **miR-24 and its target Prdx6 regulate mitochondrial ROS production and senescence of human**

231   **progenitors.**

232   miR-24 overexpression and downregulation of Prdx6 expression both led to an increased number of

233   senescent cells **(Figure 6A, B)** as compared to control group. To determine whether miR-24 and Prdx6

234   were involved in regulating ROS levels in human cells, primary myogenic progenitors were transfected

235   with miR-24 mimic, anti-miR (AM24), siRNA against Prdx6 (siPrdx6) or scrambled control RNA (Scr)

236   **(Figure 6)**. Similar to the results obtained from myogenic progenitors isolated from mice **(Figure 4),**
237   disrupted mitochondrial morphology and increased mitochondrial ROS production were detected
238   after miR-24 overexpression and Prdx6 silencing in comparison to scrambled control group **(Figure 6C-**
239   **F)**. In addition, miR-24 overexpression and Prdx6 silencing led to the presence of nuclei positive for
240   phosphorylated H2Ax, a marker of DNA damage, however this increase did not reach statistical
241   significance **(Figure 6E,F)**. Western blot analyses revealed downregulation of PRDX6 levels following
242   miR-24 overexpression (not significant) and Prdx6 siRNA (significant), however no changes were
243   detected in the levels of antioxidant protein PRDX6 (Figure 6G). Together, these data indicate that
244   miR-24 and its target Prdx6 regulate the viability, senescence and myogenic potential through
245   controlling pathways associated with mitochondrial ROS generation, DNA damage and potentially
246   unfolded protein response (UPR) with the different effects of miR-24 and Prdx6 on the levels of HSP70
247   in myogenic progenitors from adult and old mice indicating a potential mechanism underlying the
248   stronger effects of miR-24 on cells from old mice.

249

250   **DISCUSSION**
251   Muscle aging is associated with the disruption of a wide range of physiological processes affecting the
252   myocyte niche, compromising satellite cell functionality and their regenerative potential in response
253   to injury (Sannicandro *et al.* 2019).
254   Following injury, an acute increase in endogenous ROS is required to promote a pro-inflammatory
255   environment that helps with macrophage recruitment (Horn *et al.* 2017). ROS levels decrease at later
256   stages of regeneration to allow muscle hypertrophy and remodelling (Laumonier & Menetrey 2016).
257   However, this process must be tightly regulated, as chronically elevated ROS may induce irreversible
258   protein modifications, aberrant signalling, DNA damage and mutagenesis (Kidane *et al.* 2014). When
259   damage persists, cellular stressors can trigger a transient cell cycle arrest via activation of p53/p21 or
260   p16/pRB axes, which can eventually result in the induction of cellular senescence or cell death
261   programs such as apoptosis and autophagy (Vicencio *et al.* 2008).

262   This study aimed to investigate the underlying biological mechanisms of the microRNA miR-24-3p and
263   its target gene Prdx6 in muscle regeneration during aging. Our results demonstrate a transient
264   increase in miR-24 expression one day after acute injury in an *in vivo* model of skeletal muscle
265   regeneration in adult mice. miR-24 expression returned to baseline levels 7 days after injury, when
266   myoblasts stop proliferating and start differentiating to initiate tissue remodelling in mice (Grounds
267   2014). Similar to the results presented here, miR-24 expression is dynamically changed during gastric
268   metastasis progression (Li *et al.* 2016). We have also identified an upregulation of the anti-oxidant
269   Prdx6 in mouse quiescent satellite cells during aging, and confirmed Prdx6 as a direct target gene of

270 miR-24 in mice. However, changes in Prdx6 mRNA and protein levels were modest following miR-24

271 overexpression and inhibition **(Figure 4),** suggesting a fine-tuning rather than major regulator role of

272 miR-24 in controlling the levels of PRDX6.

273 Our results suggest that downregulation of miR-24 in satellite cells and concomitant upregulation of

274 its target gene Prdx6 is associated with disrupted mitochondrial network morphology, increased ROS

275 generation, an increase in the levels of phosphorylated-H2AX, a marker of DNA damage, and on a

276 phenotypic level, a decrease in cellular viability and myogenic potential, as well as increase in

277 senescence of surviving cells. Interestingly, miR-24 has been previously shown to regulate senescence-

278 associated genes (Lal et al., 2009)(Mishra et al., 2009)(Lu et al., 2018).and our results also showed

279 changes in the expression of p16, p21 and p53 in primary myogenic progenitors after miR-24

280 overexpression or Prdx6 downregulation **(Figures 3D, S3)**. Whilst on a phenotypic level, changes in

281 miR-24 levels consistently regulated cellular senescence, some differences were observed between

282 cells which have or not undergone replicative senescence in culture **(Figures 3, S3)** and cells from adult

283 and old mice **(Figures 3, S3)**. The effects of Prdx6 downregulation on cell senescence were consistent

284 in all cells suggesting that miR-24 may regulate cellular senescence through multiple targets in

285 addition to Prdx6. For example, miR-24 has been shown to increase DNA damage through regulating

286 the levels of H2AX protein or p21 (Lal et al., 2009). Moreover, according to published data, miR-24

287 regulates the expression of tumour suppressor/senescence-associated proteins differently depending

288 on the cell type and metabolic state of the cell: it reduced p16 protein levels in human diploid

289 fibroblasts and cervical carcinoma cells and it inhibited H2Ax in terminally differentiated

290 hematopoietic cells making them vulnerable to DNA damage (Lal *et al.* 2009). On the other hand, miR-

291 24 has been shown to increase p53 and p21 protein levels in different cancer cell lines (Mishra *et al.*

292 2009); and to induce p53 expression in human epithelial cells during aging and oxidative stress (Lu *et*

293 *al.* 2018). Together, these results suggest that miR-24 exerts either an inhibitory or enhancer function

294 over tumour suppressor/senescence-associated proteins depending on the cell cycle state, which is

295 consistent with our findings. Context-dependent role of miRs has been previously demonstrated, as

296 well as their dose-dependent regulation of physiological processes (Vasudevan 2012). Moreover, miR-

297 24 may regulate the expression of senescence-regulated genes *via* an upstream regulatory factor not

298 yet identified by us. The delicate balance between apoptotic, anti-apoptotic, proliferative and cell

299 cycle arrest signals will ultimately determine whether some cells successfully differentiate/self-renew

300 or, in contrast, die/become senescent.

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305   Moreover, the diverse activities of Prdx6, including peroxidase, PLA2 phospholipase and LPCAT
306   activities, mean that it could potentially regulate different metabolic signalling pathways, from cell
307   cycle, membrane repair and antioxidant response (Arevalo & Vázquez-Medina 2018; López Grueso *et*
308   *al.* 2019). One limitation of this manuscript is that it did not explore the function of Prdx6 as PLA2
309   phospholipase in the context of muscle muscle repair and ageing.

310   Another limitation of this study is the variability in the efficiency of the transfection experiments. For
311   the qPCR experiments, only samples where transfection efficiency was validated, either by an
312   increased or inhibited expression of miR-24, were taken into consideration for the quantification of
313   the target gene expression. In particular, the inhibition of miR-24 was challenging to achieve despite
314   using two different miR-24 inhibitors, which resulted in a reduced number of the independent
315   replicates used for this particular group. Likewise, the efficiency in the transfection of the cells used
316   for the immunofluorescence experiments might also be affected. Another limitation to be considered
317   is the use of solely one technique for the assessment of ROS generation and oxidative stress. Future
318   studies should corroborate these findings using additional approaches. Despite using two
319   independent assays to detect cellular senescence, SA-β-gal results should be interpreted with caution,
320   as increased intensity of the staining in some cells did not always correlate with an extension of the
321   cytoplasm, which is a well-known characteristic of senescent cells. Therefore, SA-β-gal staining and
322   higher levels of tumour suppressor proteins may not always indicate a permanent cell cycle arrest, but
323   probably a stress-induced transient cell cycle arrest that might trigger alternative processes such as
324   apoptosis or autophagy. This assumption fits well with our data, where miR-24 overexpression and
325   Prdx6 downregulation lead to cell death and increased senescence. Alternatively, the cells
326   overexpressing miR-24 or cells with downregulated levels of Prdx6 may enter irreversible senescence
327   and the final post-senescent stage of cell death (Gamez et al., 2019). Cell senescence and cell death
328   share common factors and both have been shown to be interdependent in certain scenarios (Gamez
329   et al., 2019). Our data indicate that overexpression of miR-24 and downregulation of Prdx6 are
330   associated with changes in mitochondrial morphology, increase in mitochondrial ROS generation and
331   increased levels of phospho-H2Ax **(Figures 3,6)**. It has been shown that following DNA damage, cells
332   undergo a temporary cell cycle arrest in an attempt to repair their DNA; if the DNA damage is
333   unresolved, cells can undergo apoptosis or become senescent; in case of increased damage in
334   senescent cells, senescent cells may undergo cell death (Gamez et al., 2019).

335 Noteworthy, myogenic progenitors isolated from mouse were from males whereas human samples

336 were retrieved from female donors. Several studies have shown biological differences between rodent

337 males and females in the development of sarcopenia and efficiency in muscle regeneration, as well as

338 in the global expression of microRNAs in human skeletal muscle (Maher *et al.* 2009; Kob *et al.* 2015).

339 It is thus important to point out that the altered expression of genes in satellite cells and muscle

340 progenitors shown in this study might be sex-specific in addition to species-associated differences.

341 In summary, our results identify a role for miR-24-3p through inhibition of Prdx6 in satellite cells during

342 aging which may play a key role in early stages of skeletal muscle regeneration after acute injury,

343 through controlling adaptive redox and apoptotic and senescence signalling pathways. Moreover, our

344 findings show that miR-24 and Prdx6 regulation of myogenic progenitor phenotype is more

345 pronounced in cells from old mice, likely due to miR-24 regulation of additional to Prdx6 target genes,

346 such as p21. This mechanism may not be as strongly conserved in mice as in humans, as the effects

347 of miR-24 regulated ROS, myoblast viability, differentiation and senescence was more pronounced in

348 myoblasts from adult humans. This is not surprising, as miR-24 binding site in Prdx6 resides at the

349 3'UTR of the human Prdx6 transcript, whereas in mice, this site has a weaker interaction at the 5'UTR

350 of the Prdx6 transcript. The role of miR-24 in the regulation of muscle regeneration requires further

351 *in vivo* studies given the subtle differences in the phenotype induced by miR-24 on myogenic

352 progenitors from adult and old mice, as these could be further exacerbated through changes in the

353 satellite cell niche during aging.

354 We propose that changes in miR-24 and Prdx6 levels in satellite cells during aging represent an

355 adaptive response to aging aimed at improving cellular viability and myogenic potential and decrease

356 of cellular senescence through regulating mitochondrial ROS generation and potentially associated

357 with it DNA damage.

358

359 **EXPERIMENTAL PROCEDURES**

360 **Reagents.**

361 All reagents are listed in supplementary tables.

362

363 **Mouse samples**

364 All experiments described herein received the ethical approval from The University of Liverpool

365 Animal Welfare and Ethical Review Body (AWERB) and were performed in accordance with UK Home

366 Office guidelines under the UK Animals (Scientific Procedures) Act 1986. All mice were male wild-type

367 C57Bl/6 from Charles River (Margaret), maintained under SPF conditions and fed *ad libitum* and

368 maintained under barrier on a 12 hours light/dark cycle. For muscle regeneration, tibialis anterior

369   muscle was injured by intramuscular injection of barium chloride (1.2% in saline). Tissue was collected

370   1, 7, 14, or 21 days after injury. Muscle was snap-frozen in liquid nitrogen and stored at -80°C. Muscle

371   progenitor cells and satellite cells were directly isolated from fresh lower limbs muscles (extensor

372   digitorum longus, tibialis anterior, gastrocnemius, quadriceps and soleus). For each experiment, n = 3-

373   7 independent replicates per group were used. Young: 6-12 weeks old; adult: 6-8 months old; old: 20-

374   24 months old. For miR-24 and Prdx6 expression in FACS-sorted satellite cells: adult: 1-8 months old;

375   old: 20-24 months old.

376   **Human samples**

377   All experiments described herein involving human samples were performed according to good

378   practice guidance and in accordance with The University of Liverpool, University Hospital Aintree

379   Hospital and South West Wales Research Ethics Committee (Approval No: 13/WA/0374). The

380   University of Liverpool acted as the ethics sponsor for this study. All the donors had given informed

381   consent for enrolment in this study. Muscle biopsies were obtained from foot surgeries (extensor

382   digitorum brevis, tibialis anterior or abductor hallucis muscles) of female patients treated for Hallux

383   Valgus, with an average age of 33 ± 6.78 years old and a Body Mass Index (BMI) < 25. For each

384   experiment, and due to limitations in sample availability, both human primary myogenic progenitors

385   isolated from female donors (n = 2-5 per experiment) and commercialised human primary skeletal

386   muscle progenitors (ThermoFisher Scientific, n = 1-2 per experiment) were used. For all the

387   experiments n = 3-7 independent replicates per group, unless stated otherwise.

388   **Satellite cell isolation**

389   Satellite cells were isolated using FACS as previously described (Yi & Rossi 2011; Soriano-Arroquia *et*

390   *al.* 2016). Briefly, skeletal muscle was isolated from the hind limbs of C57Bl/6 wild type male mice and

391   enzymatically digested with 1.5 U mL-1 collagenase D, 2.4 U mL-1 dispase II and 2.5 mM $CaCl_2$. Cells

392   were then dissolved in sterile FACS buffer (2% horse serum in DPBS), filtered through a 40μm cell

393   strainer and stained with conjugated antibodies in the dark for 30 minutes on ice. Doublets were

394   discriminated and haematopoietic and endothelial cells (PE-CD31+/CD45+) were excluded from the

395   sorting gates. Satellite cell population was isolated as BV421-CD34+, Alexa647-Alpha7Integrin+, FICT-

396   Sca1-, PE-CD31-, PE-CD45- and eFluor780-Viability- dye. Sorting was performed at 4°C and samples

397   were collected in growth media (high-glucose DMEM supplemented with 10% FBS, 1% L-glutamine

398   and 1% penicillin/streptomycin). Sorted cells were immediately centrifuged and resuspended in Qiazol

399   (Qiagen) for total RNA isolation.

400   **RNA isolation**

401   For RNA isolation, cells were collected 48 hr after transfection. Total RNA from sorted cells was

402   isolated using miRNeasy Mini Kit (Qiagen). Total RNA from primary cells was isolated using

403   TRIzol/Chloroform standard protocol. After isolation, if necessary samples were purified using ethanol

404   and sodium acetate. RNA concentration and quality were assessed using Nanodrop 2000.

405   **Real-Time qPCR**

406   cDNA synthesis and Real-Time qPCR were performed as previously described (Soriano-Arroquia *et al.*

407   2016). Briefly, cDNA synthesis was performed from 500ng of RNA (for mRNA) or 100ng of RNA (for

408   microRNA) using SuperScript II (ThermoFisher) or miRscript RT kit II (Qiagen), respectively. SYBR Green

409   Mastermix (Qiagen) or SsoAdvanced Universal SYBR Green Supermix (BioRad) or FastSybrGreen

410   (Thermo Fisher; 4385610) were used for Real-Time quantitative PCR. Relative expression to β-actin,

411   18S, S29, β -2 microglobulin (mRNA) or Snord-61 (microRNA) was calculated using delta C_t method

412   (Soriano-Arroquia *et al.* 2016).

413   **Isolation of primary muscle progenitor cells from mouse and human skeletal muscles**

414   The isolation of human and mouse primary muscle progenitor cells was performed as previously

415   described (Soriano-Arroquia *et al.* 2017). Briefly, skeletal muscle tissue was enzymatically digested

416   with 1.5 U mL^{-1} collagenase D, 2.4 U mL^{-1} dispase II and 2.5 mM $CaCl_2$. Digested muscles were

417   harvested on culture dishes coated with 10μg mL^{-1} laminin and cultured with F-12 media

418   complemented with 20% FBS, 10% horse serum, 1% L-glutamine, 1% penicillin/streptomycin and 2.5

419   ng/mL bFGF (Recombinant Human FGF-basic). Human cells were grown in high-glucose DMEM

420   supplemented with 20% FBS, 10% horse serum, 1% L-glutamine and 1% penicillin/streptomycin, and

421   mouse cells were grown in high-glucose DMEM supplemented with 10% FBS, 1% L-glutamine and 1%

422   penicillin/streptomycin. For differentiation, both human and mouse primary muscle progenitor cells

423   were cultured in high-glucose DMEM supplemented with 2% horse serum, 1% L-glutamine and 1%

424   penicillin/streptomycin.

425   **Transfections and immunostaining**

426   All cells in main figures were isolated from 6 month (adult) or 24 month (old) old mice. Cells used in

427   experiments presented in supplementary data were isolated from mice aged 1-8 months (young and

428   adult) or 20-24 months (old) . Transfections of primary cells were performed as previously described

429   (Soriano-Arroquia *et al.* 2017). Briefly, primary cells were transfected with 100nM of miR-24-3p mimic,

430   100nM of miR-24-3p inhibitor, 100nM of scrambled control or 100nM of siRNA against Prdx6 using

431   Lipofectamine 2000 transfection reagent (ThermoFisher). Cells were used at P4-P7. Cells were plated

432   at either 80% confluency (differentiation, qPCR, western blotting), or 50% confluency (viability,

433   proliferation, senescence, MitoTracker and MitoSox staining). Culture media was changed to

434   differentiation media (high-glucose DMEM complemented with 2% HS, 1% P/S, 1% α-glutamine) 6

435   hours after transfection. No media was changed until collection or staining of the cells. Control cells

436   were transfected with scrambled control. Immunostaining was performed 48 hr (Ki67 staining), 4 days

437   (viability assay), 7 days (SA-β-galactosidase staining) and 7-10 days (MF 20 staining) after transfection.

438   RNA and protein were isolated 48 hr after transfection. Staining for MF 20 , SA-β-galactosidase, MF

439   20, Ki67 and viability assay was performed as previously published methods (Soriano-Arroquia *et al.*

440   2017). Fluorescent SA-β-galactosidase, Mitotracker Red, Mitosox and DNA damages were performed

441   using Cell Event Cell senescence kit, MitoSox Red, MitotTracker Red CM-H2Xros and HCS DNA damage

442   kits (Thermo Fisher) according to manufacturer's protocols.

443   For Western blotting cells were lysed in RIPA buffer and protein concentrations were calculated using

444   Bradford reagent with BSA as standards. For immunoblotting 20µg (mouse) or 15µg (human) of

445   protein was loaded on a 10-14% polyacrylamide gels. Following gel electrophoresis samples were

446   transferred onto nitrocellulose membrane and total protein was stained using Ponceau S. Following

447   washing of the membrane with TBS-T, membranes were blocked for 1h at room temperature using

448   either 5% BSA or milk, membranes were washed 3 x 10 min in TBS-T and incubated overnight at 4C

449   with primary antibodies (see Suppl Tables). Membranes were washed and incubated with secondary

450   antibodies goat anti-rabbit and goat anti-mouse (Li-Cor Biosciences) and images were obtained using

451   Odyssey Fc imaging system (Li-Cor). Quantification of blots and normalisation was performed using

452   Image Studio Lite (Li-Cor).

miR:target binding reporter assay

454   5'UTR of Prdx6-202 transcript regions with either the wild-type or mutated miR-24-3p target sites

455   were synthesized using GeneArt service (Thermo Scientific). The wild type or mutated sequences were

456   subcloned into a GFP TOPO vector (Thermo Scientific). C2C12 myoblasts were cultured in 96-well

457   plates and transfected using Lipofectamine 2000™ (Thermo Scientific) with either 200ng of the wild

458   type or mutant sensor and with either 100nM of the miR scrambled control or 100nM miR-24 mimic.

459   Each experiment was carried out using at least two independent plasmid preparations in triplicate.

460   GFP fluorescence was measured 48 hr following transfections using FLUOstar Optima microplate

461   reader (BMG Labtech).

Image analysis

463   Cells were semi-automatically quantified using Fiji and ImageJ (Schindelin et al., 2012) followed by

464   manual correction. At least 3-6 random images from different fields of view per biological sample at

465   10x magnification (100x total magnification) were captured. The only exception of this rule was for

466   human β-galactosidase analysis, in which a complete tiled field of view image was analysed per

467   biological sample. For myogenic differentiation analyses, fusion index is shown as the percentage of

468   nuclei contained within myotubes to the total number of nuclei in each field of view. For the

469   quantification of senescent cells, cells were counted manually (only cells showing intense blue staining

470   were classified as senescent) or β-galactosidase activity values (BGAVs) were calculated as previously

471   described by Shlush et al. (Shlush *et al.* 2011). Cells with a BGAV ≥ 15 were considered as highly

472   senescent (SA-βgal^{high}, characterised by an intense blue staining); cells with a BGAV between 5-14 both

473   inclusive were considered as low senescent (SA-βgal^{low}, characterised by an light blue staining); and

474   cells with a BGAV < 5 were considered as non-senescent (SA-βgal^{non}, no blue staining). All the

475   immunostaining quantifications were manually curated. Images were captured using Nikon Eclipse Ti-

476   E inverted confocal microscope (supplementary data) and Carl Zeiss Axiovert 200 inverted microscope

477   (for SA-βgal staining) or EVOS M5000 and EVOS M7000 (Thermo Fisher, main figures).

478   **Statistical analysis**

479   Details of the statistical analyses used per experiment are described in the corresponding figure

480   legend. T-test or Mann-Whitney (qPCR data which is not normally distributed) test were performed

481   for the analysis of statistical differences between two groups as stated. One way or two-way

482   A.N.O.V.A. followed by Tukey's multiple comparison test, or Kruskal-Wallis followed by Dunn's

483   multiple comparison test with 95% Confidence Interval was performed to compare more than two

484   groups as indicated where data was not normally distributed. p-value < 0.05 was considered

485   statistically significant. All analysis was performed on raw (not normalised) data. For the transfection

486   experiments, individual values representing the same independent biological replicate have been

487   matched with a dotted line. Statistical analysis were performed using GraphPad Prism version

488   8.4.2/9.0.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

489   **Gene ontology**

490   A list of human and mouse miR-24 predicted targets were obtained from TargetScanHuman 6.2 (Lewis

491   *et al.* 2005; Grimson *et al.* 2007; Agarwal *et al.* 2015). Human and mouse miR-24:targets network

492   interaction and GO analyses were performed using Cytoscape v.3.8.0 (Shannon *et al.* 2003) and

493   ClueGO v.2.5.6 plugin for Cytoscape (Bindea *et al.* 2009), respectively. Details of the statistics used for

494   ClueGO are specified in the corresponding figure legend. Generally: Enrichment/Depletion (Two-sided

495   hypergeometric test); Minimum p-value cut-off = 0.01; Correction Method = Bonferroni step down;

496   Min GO Level = 5; Max GO Level = 8; Kappa Score Threshold = 0.4-0.55.

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508   **CONFLICT OF INTERESTS**

509   The authors declare no conflict of interests associated with this manuscript.

510   **AUTHOR CONTRIBUTIONS**

511   AS, JG, QX, KW, BMcD performed the experiments; AS, JG, DB, QX, BMcD and KW performed data

512   analyses; all authors contributed to experimental design, statistical analyses and manuscript

513   preparation.

514   **Data availability statement**

515   Source data for microscopy images and FACS data have been deposited into Mendeley data set:
516   doi:10.17632/g7593chtxy.1

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640

641   **FIGURE LEGENDS**

642   **Figure 1.** miR-24 expression is affected by muscle injury and aging. **(A)** miR-24 is predicted to target

643   genes and processes associated with redox balance in humans. Gene ontology (GO) analysis was

644   performed by ClueGO plugin for Cytoscape (v.2.5.6). Statistical test used for ClueGO:

645   Enrichment/Depletion (Two-sided hypergeometric test). p-value cut-off = 1.0E-4. Correction Method

646   = Bonferroni step down. Min GO Level = 5; Max GO Level = 8; Number of Genes = 16; Min Percentage

647   = 4.0; Kappa Score Threshold = 0.4. Only targets involved in 'cellular response to oxidative stress' are

648   shown. **(B)** The percentage of mouse satellite cells decreases during aging (n = 4-7, R^2 = 0.9484). **(C-E)**

649   Myogenic progenitors from old mice are less viable, have decreased myogenic potential and display

650   increased senescence (n = 3-6, two-tailed unpaired Student's t-test). **(F)** The accumulation of ROS

651   assessed using the CM-H_2DCFDA assay; mean fluorescence intensity shown. **(G)** Increased production

652   of mitochondrial ROS detected by MitoSox Red in mouse myogenic progenitors during aging (n = 3,

653   upaired t-test). **(H)** qPCR showing decreased expression of miR-24 in mouse satellite cells during aging.

654   Expression relative to Snord61 is shown (n = 5-7, Mann-Whitney test). **(I)** Diagram representing tissue

655   collection points following TA injury using BaCl$_2$. **(J)** qPCR of miR-24 in the TA after injury. Expression

656   relative to Snord61 is shown (n = 3, two-way A.N.O.V.A followed by Tukey's multiple comparison test

657   with 95% Confidence Interval). Young: 6-12 weeks old; adult: 6-8 months old; old: 20-24 months old.

658   **(K)** Representative images of H&E and WGA staining indicating the extent of muscle damage following

659   BaCl2 injury of the gastrocnemius muscle from adult and old muscle. Scale bars: 200 μm. For miR-24

660   qPCR in satellite cells: adult: 1-8 months old; old: 20-24 months old. p-value < 0.05 was considered as

661   statistically significant (*). Error bars show S.E.M.

662   **Figure 2.** miR-24 regulates viability and differentiation of myogenic progenitors during aging. **(A, D)**

663   Myogenic progenitors isolated from adult and old mice were transfected with miR-24 or AM24. Cells

664   transfected with scrambled control were used as control. Scale bars: 300μm. **(A)** Viability assay shows

665   viable (green), apoptotic (yellow) and necrotic (red) cells. **(B)** miR-24 overexpression and

666   downregulation of its target Prdx6 resulted in significant decrease in % viable cells from old mice. **(C)**

667   MF 20 (anti-myosin heavy chain; green) and DAPI (blue) immunostaining were performed for

668   myogenic differentiation and nuclei identification, respectively. **(D)** Overexpression of miR-24

669   significantly affected the differentiation of myogenic progenitors from adult mice, inhibition of miR-

670   24 target Prdx6 inhibited myogenic differentiation of muscle progenitors from muscle of adult and old

671   mice, whereas miR-24 inhibition led to improved myogenesis of muscle progenitors from muscle of

672   adult and old mice. All panels: n = 3-4, One-Way A.N.O.V.A followed by Tukeys multiple comparison

673   test. Adult: 6 months old; old: 24 months old. p-value < 0.05 was considered as statistically significant

674   (*p < .05). Error bars show S.E.M.

Figure 3. miR-24 overexpression and downregulation of its target gene Prdx6 lead to increased number of senescent cells through increased mitochondrial ROS generation and DNA damage. **(A)** Myogenic progenitors isolated were transfected with miR-24 mimic (miR-24) or anti-miR (AM24). Cells transfected with scrambled control were used as control. SA-βgal staining (B) or fluorescent SA-βgal staining (C) was performed for the assessment of senescent cells (blue). Scale bars: 300µm. **(B, C)** miR-24 overexpression and inhibition of Prdx3 resulted in a higher % of senescent cells in the adult or both mice, respectively; n = 3, One-Way A.N.O.V.A followed by Tukeys). **(D)** miR-24 and Prdx6 overexpression/inhibition was associated with increased p53 and p16 expression in myogenic progenitors from old mice; expression relative to 29S is shown; One-Way A.N.O.V.A followed by Tukeys. **(E)** MitoTracker staining of myogenic progenitors from adult and old mice indicates dysregulation of mitochondrial networks following miR-24 overexpression and Prdx6 downregulation. **(F)** Mitosox Red staining indicates increase in mitochondrial ROS production following miR-24 overexpression and Prdx6 downregulation. **(G)** Overexpression of miR-24 and inhibition of Prdx6 led to an increase in the presence of nuclei stained for phosphorylated H2Ax, a marker of DNA damage. Scale bars: 75µm **(H)** Quantification of Mitosox Red staining and phosphor-H2Ax staining indicate increase in ROS generation following miR-24 overexpression and Prdx6 downregulation in myogenic progenitors from adult and old mice and increase in DNA damage marker in muscle of old mice following miR-24 overexpression. miR-24 and siPrdx6 expression manipulation did not result in changes in H2ax expression (qPCR). Adult: 6 months old; old: 24 months old. p-value < 0.05 was considered as statistically significant (*p < .05;). Error bars show S.E.M.

Figure 4. miR-24 fine-tunes the levels of Prdx6 levels through its target site. **(A)** qPCR showing Prdx6 expression in mouse satellite cells during aging. Expression relative to beta-actin is shown. Adult: 1-8 months old. Old: 20-24 months old (n = 5, Mann-Whitney test). **(B)** Putative miR-24-3p seed sequence in the 5' UTR of Prdx6 gene (highlighted in grey). Mutated seed sequence used for 5'UTR microRNA:mRNA target interaction is shown. Mutation is shown in red. **(C)** miR-24 directly regulates the expression of Prdx6. GFP-Prdx6 5'UTR sensor construct containing the wild type or mutated seed sequence were transfected into C2C12 myoblast cell line and co-transfected with miR-24 or scrambled control (Scr). The wild type construct transfected with miR-24 mimic shows less GFP fluorescence intensity compared to the scrambled control, but not in the mutated construct. (Representative data shown; n = 3, two-tailed unpaired Student's t-test). **(D, E)** qPCR showing the expression of Prdx6 after microRNA mimic or antagomiR (AM24) transfection in primary myogenic progenitors isolated from adult **(D)** and old mice **(E)**. Expression relative to 18S is shown. Adult: 6-8 months old; old: 20-24 months old (n = 3-7, Kruskal-Wallis test followed by Dunn's multiple comparisons test with 95% Confidence Interval). **(F, G)** Western blotting indicating changes in PRDX6 levels following miR-24

709   overexpression or inhibition suggesting miR-24 fine-tuning the levels of PRDX6 rather than being a

710   master regulator of PRDX6 levels. miR-24 overexpression and inhibition or downregulation of PRDX6

711   had no effect on the levels of antioxidant protein PRDX3, n=3. One-Way A.N.O.V.A followed by Tukey's

712   multiple comparison. For all the figures unless stated otherwise: adult: 6 months old; old: 24 months

713   old. p-value < 0.05 was considered as statistically significant (*p < .05). Error bars show S.E.M.

714   **Figure 5.** miR-24 and Prdx6 regulate viability and differentiation of human primary myoblasts. **(A, B)**

715   Human primary myogenic progenitor cells isolated from adults were transfected with miR-24 or AM24

716   or siRNA for Prdx6. Cells transfected with scrambled RNA were used as control (Control). MF 20 (anti-

717   myosin heavy chain; green) and DAPI (blue) immunostaining were performed for myogenic

718   differentiation and nuclei identification, respectively. Viability assay was performed with ethidium

719   bromide and acridine orange for the assessment of viable (green), apoptotic (yellow) and necrotic

720   (red) cells. SA-βgal staining was performed for the assessment of senescent cells (blue). Scale bars:

721   200μm. **(C, D)** miR-24 overexpression and downregulation of Prdx6 resulted in decreased number of

722   viable cells, as well as thinner myotubes containing fewer nuclei (n = 3, One-Way A.N.O.V.A followed

723   by Tukeys multiple comparison). p-value < 0.05 was considered as statistically significant (*p < .05).

724   Error bars show S.E.M. Scale bars: 300μm.

725   **Figure 6.** miR-24 and its target regulate senescence of human myogenic progenitors through

726   mitochondrial ROS production. **(A)** Human myogenic progenitors isolated were transfected with miR-

727   24 mimic (miR-24) or anti-miR (AM24). Cells transfected with scrambled control were used as control.

728   SA-βgal staining (B) or fluorescent SA-βgal staining (C) was performed for the assessment of senescent

729   cells (blue). Scale bars: 300μm. **(B)** Quantification of senescent cells indicates increase in the

730   proportion and number of senescent cells following miR-24 overexpression and downregulation of

731   Prdx6 expression. **(C)** MitoTracker Red staining indicates changes in mitochondrial morphology

732   following miR-24 overexpression and downregulation of siPrdx6 levels. (D, F) Mitosox Red staining

733   indicates increased mitochondrial ROS levels following miR-24 overexpression and downregulation of

734   Prdx6 expression. Scale bars: 75μm. **(E, F)** No significant changes were detected in DNA damage

735   marker, phosphorylated H2Ax. **(G)** Western blot indicating miR-24 fine-tuning the expression of Prdx6

736   rather than being a master regulator of its expression. No changes were detected in the levels of

737   antioxidant protein levels: PRDX3 following changes in miR-24 or Prdx6 expression. One-way

738   A.N.O.V.A. followed by Tukey's multiple comparison test with 95% Confidence Interval). p-value < 0.05

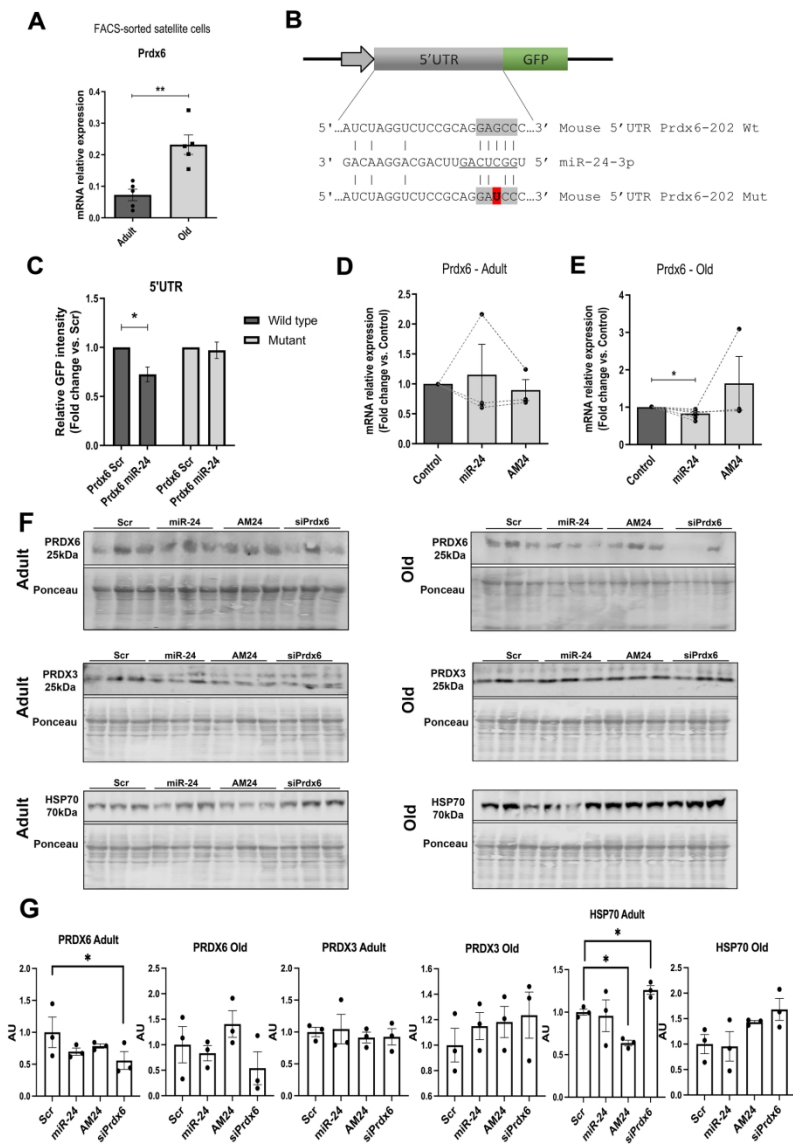739   was considered as statistically significant (*p < .05). Error bars show S.E.M.
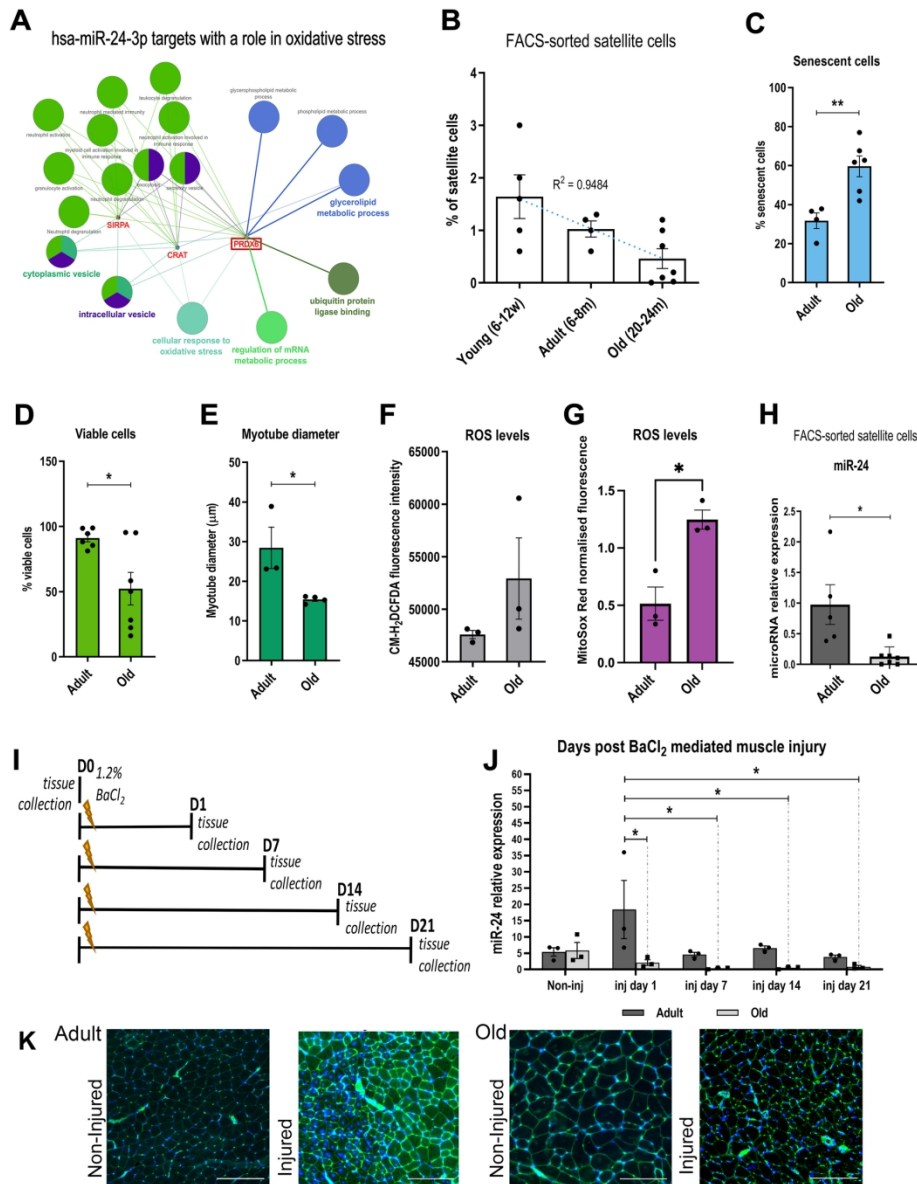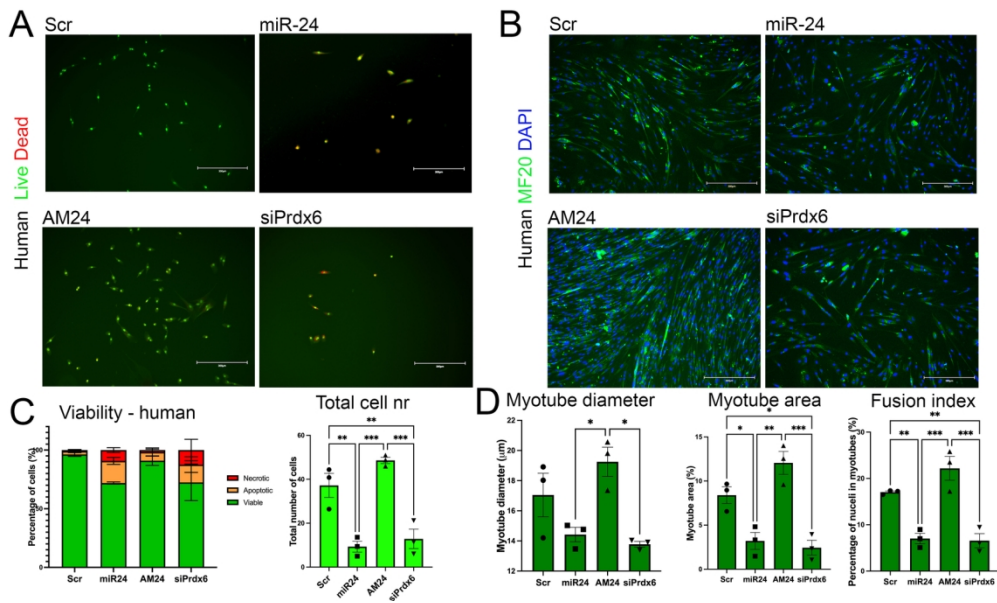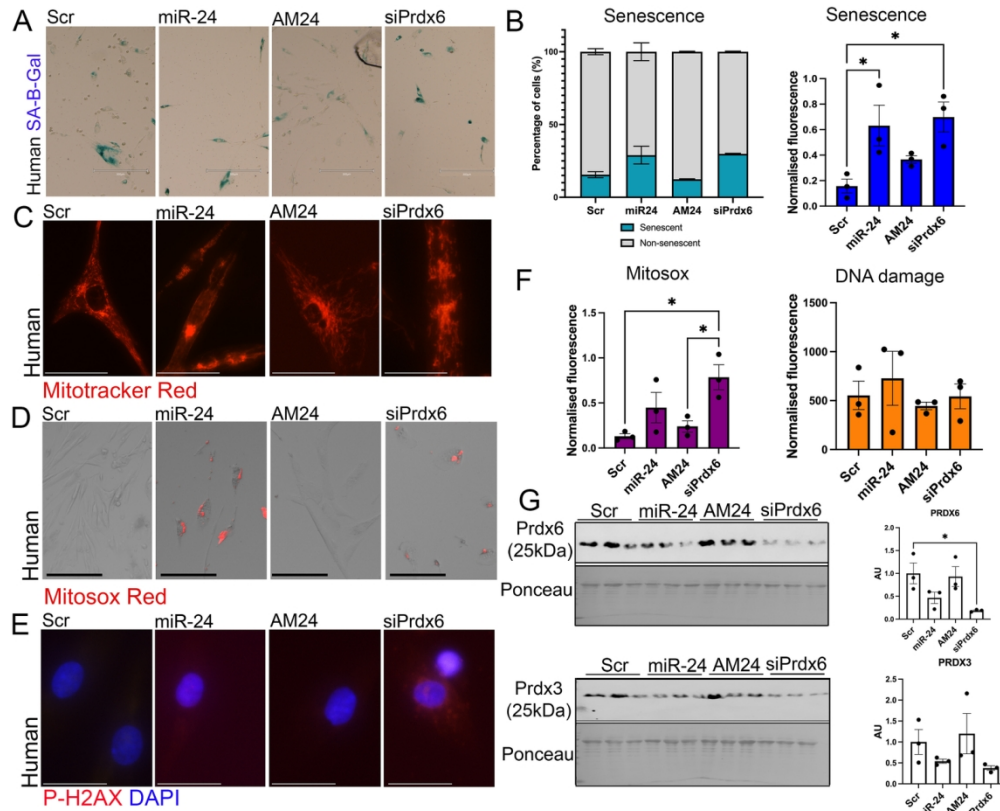
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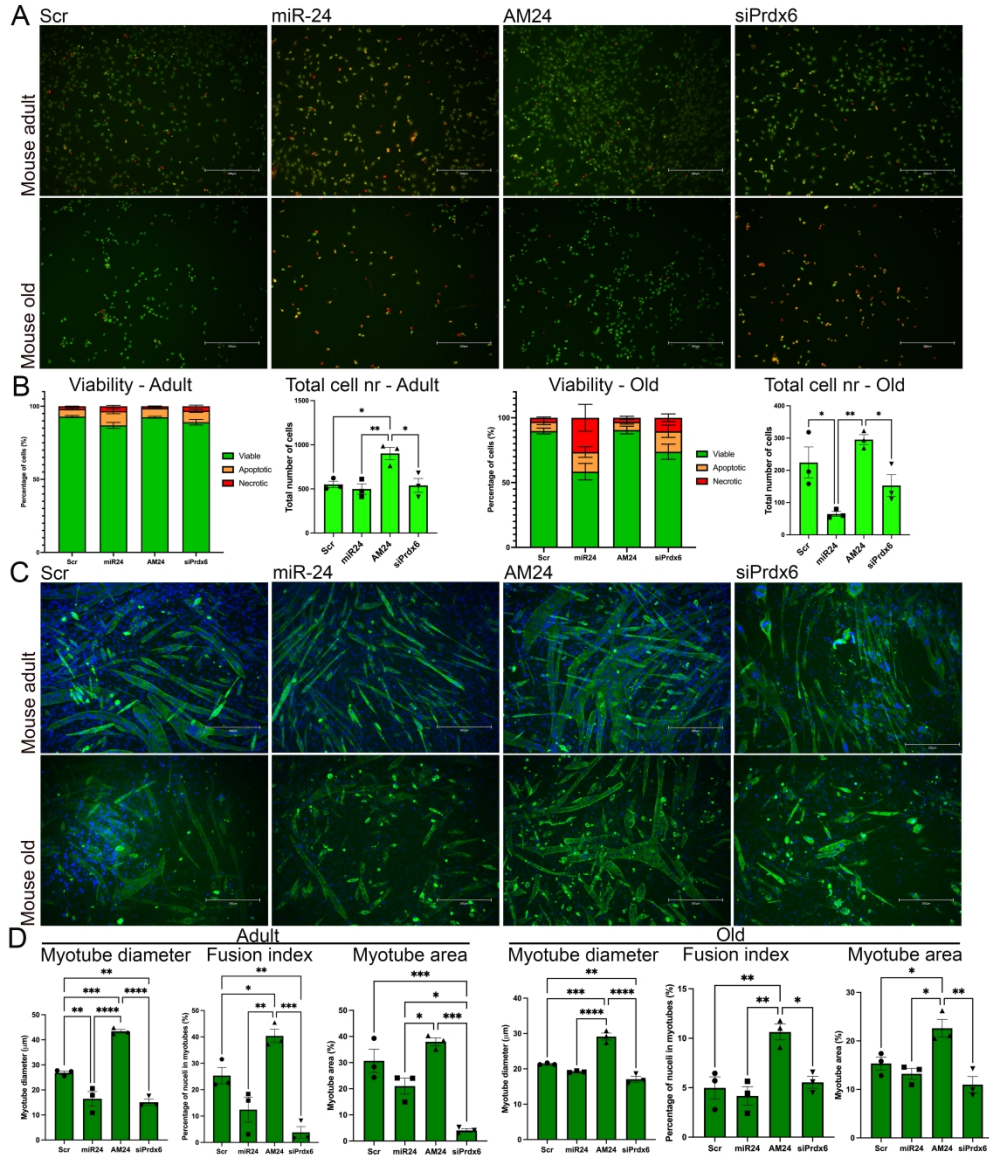
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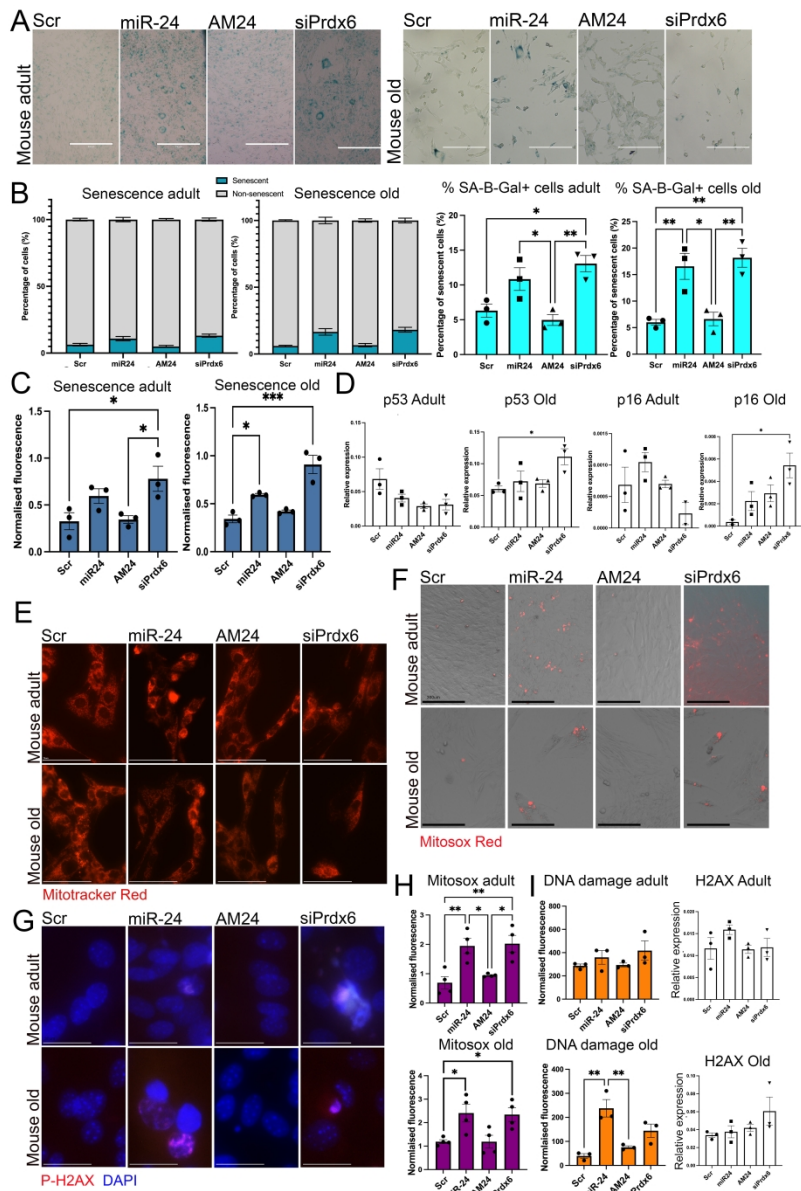
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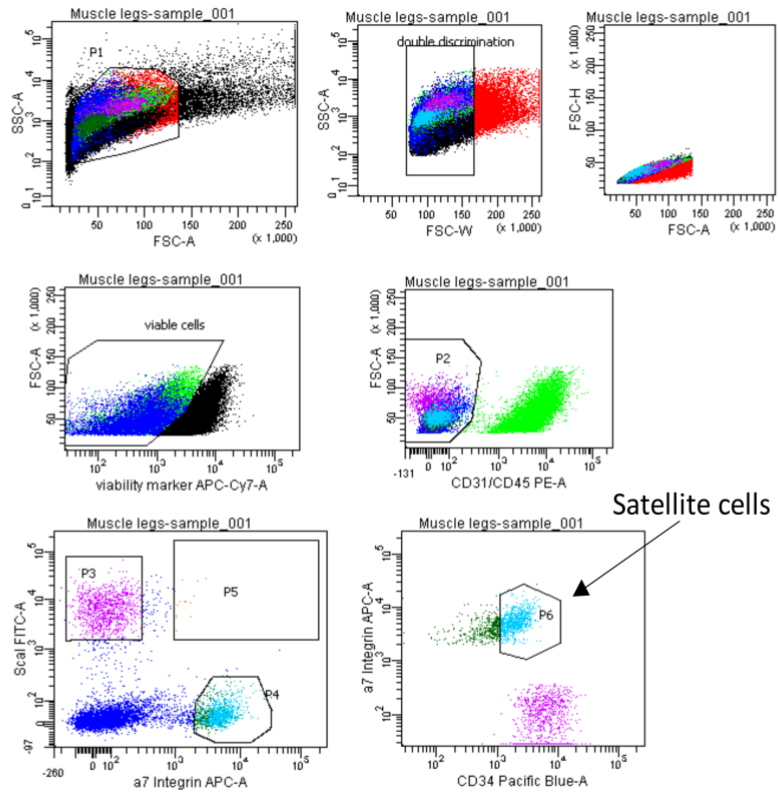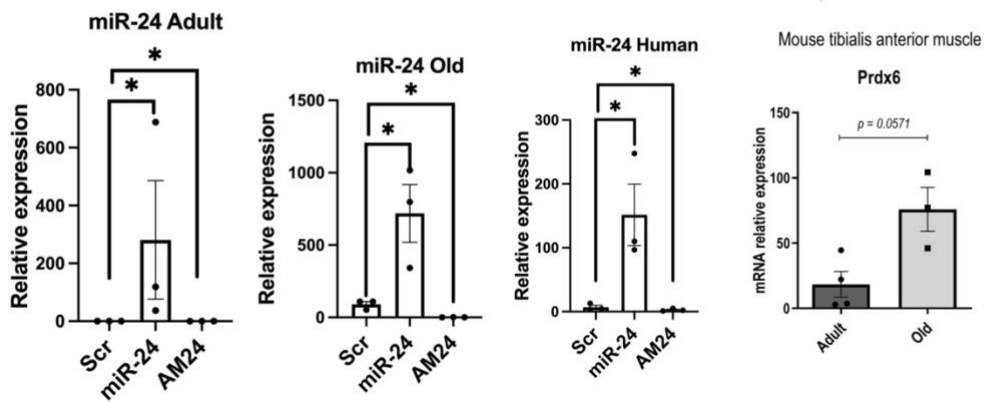
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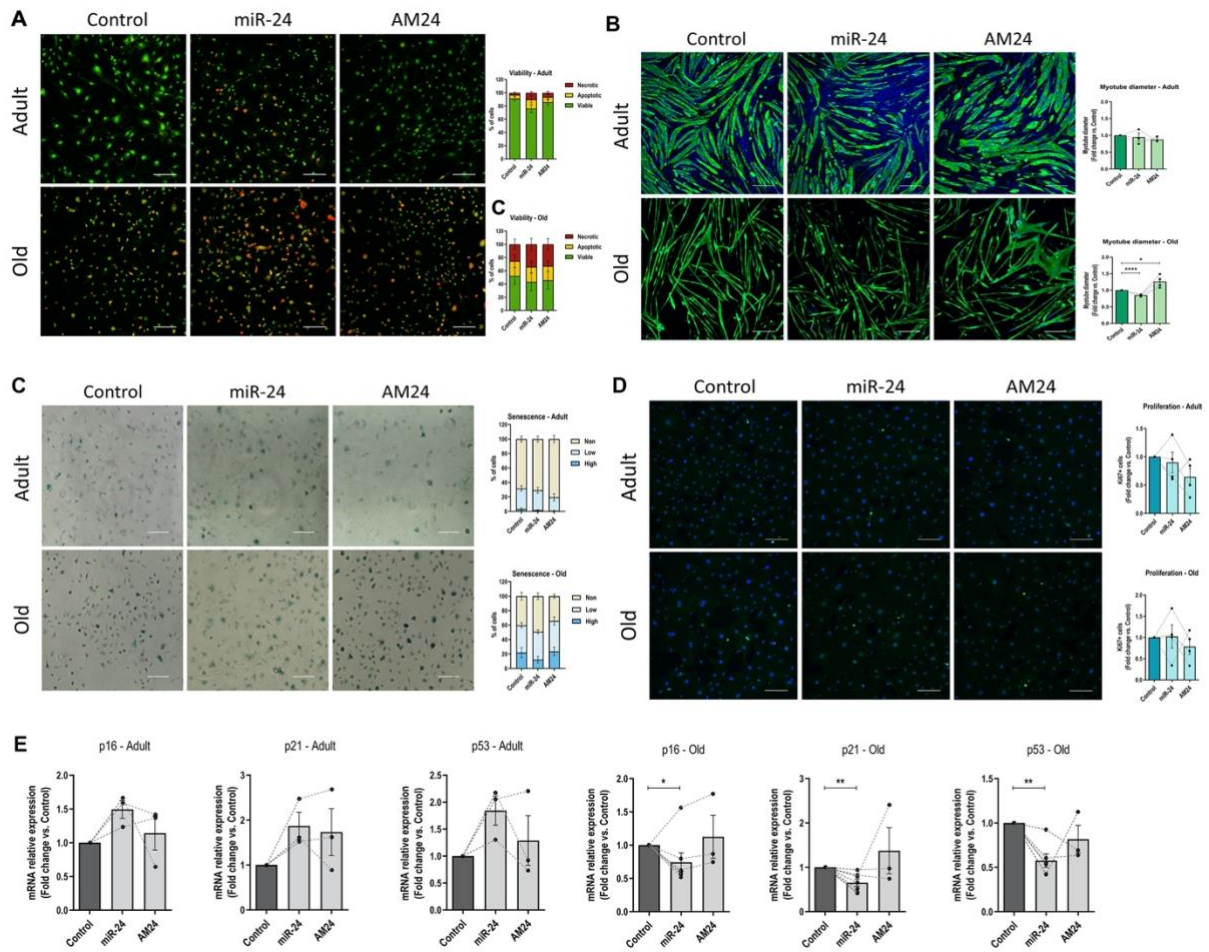136x112mm (300 x 300 DPI)

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Fig. S1

Fig. S2

Figure S3.

Human primary myoblasts
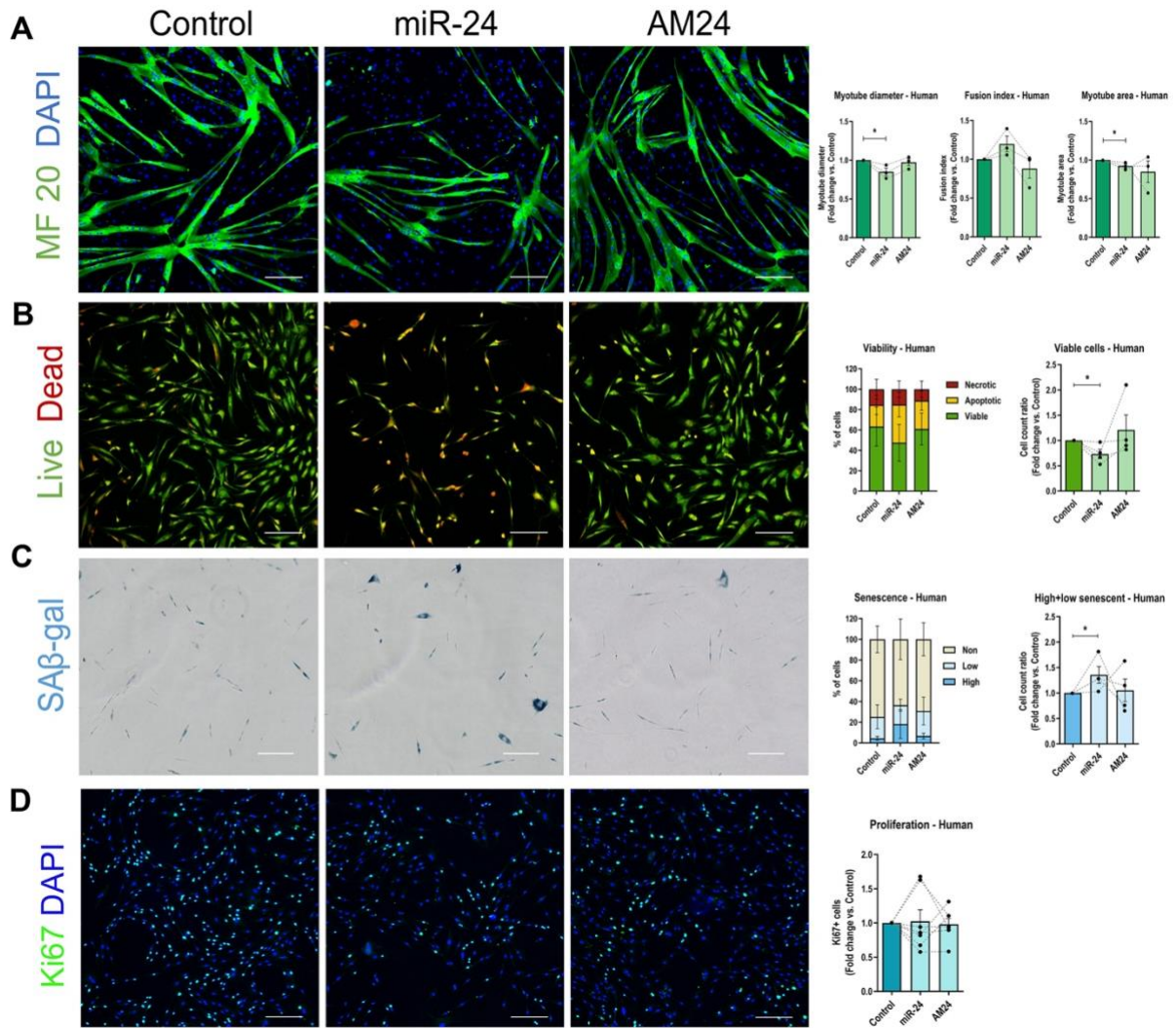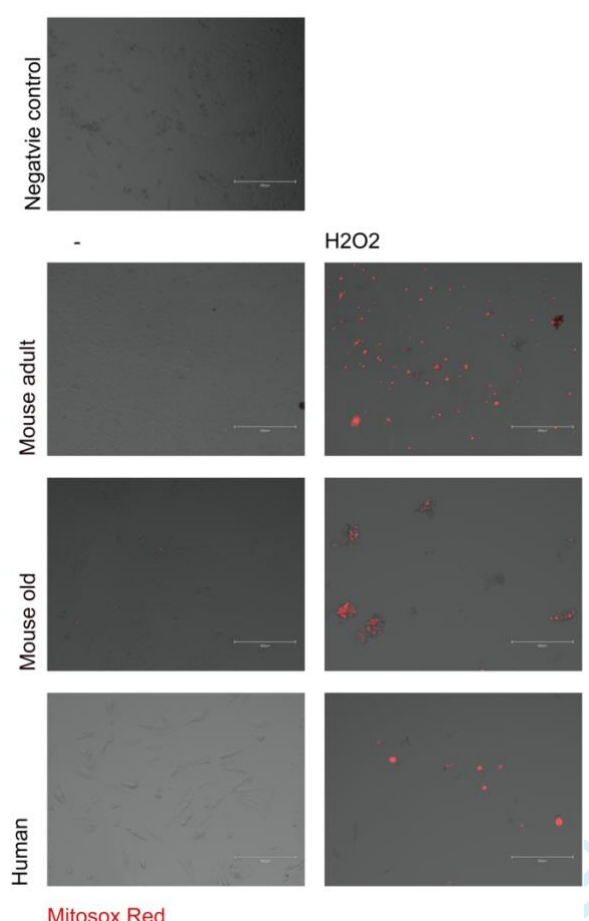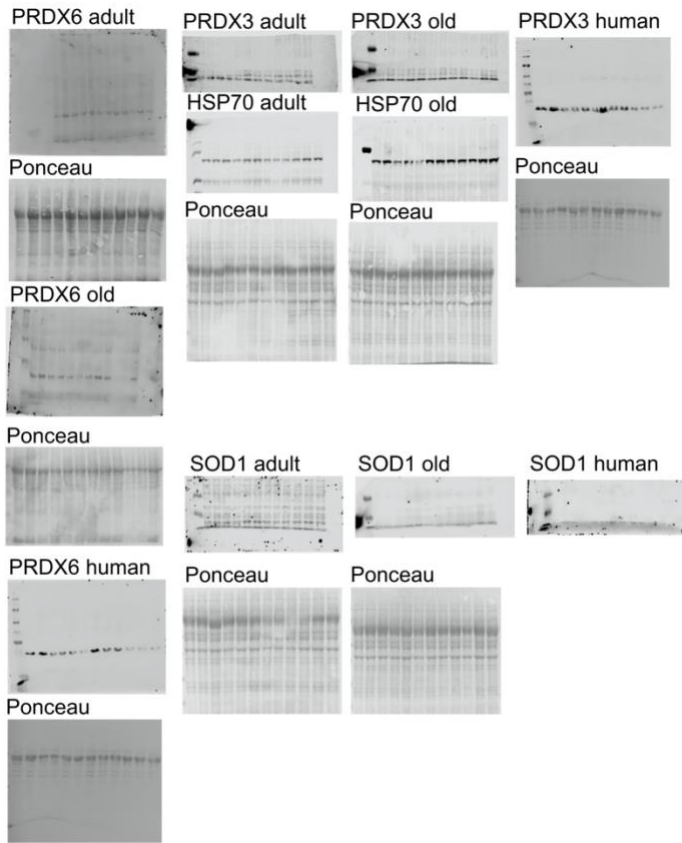

Figure S4.

Figure S5.

Figure S6.

Supplementary figure legends:

Figure S1. Illustration showing the isolation of quiescent satellite cells by FACS (fluorescence activated cell sorting). The satellite cells population was positive for CD34, highly-positive for alpha7-integrin, and negative for Sca1, CD31 and CD45.

Figure S2. (A) qPCR showing the expression of miR-24 after microRNA mimic or antagomiR (AM24) transfection in primary myogenic progenitors isolated from adult and old mice. Expression relative to Snord61 is shown (n = 3-7, adult: 6-8 months old; old: 20-24 months old). F-test compared to control. p-value < 0.05 was considered as statistically significant (# p < .05; ## p < .01; ### p < .001; #### p < .0001). Error bars show S.E.M. **(B)** qPCR showing Prdx6 expression in mouse tibialis anterior muscle during aging. Expression relative to ß-2 microglobulin is shown (n = 3-4, adult: 6-8 months old; old: 20-24 months old). Mann-Whitney test (* < .05; **p < .01; ***p < .001). Error bars show S.E.M.

Figure S3. miR-24 regulates function of myogenic progenitors during aging. (A) Viability assay shows viable (green), apoptotic (yellow) and necrotic (red) cells. miR-24 overexpression resulted in decreased viable cells from both adult and old mice. miR-24 inhibition also affected viability of cells isolated from old mice (n = 6-7, two-tailed unpaired Student's t-test compared to control). (B) MF 20 (anti-myosin heavy chain; green) and DAPI (blue) immunostaining were performed for myogenic differentiation and nuclei identification, respectively. miR-24 overexpression resulted in decreased myotube diameter whereas miR-24 inhibition resulted in bigger myotubes in myogenic progenitors from old mice compared to control (n = 3-4, two-tailed unpaired Student's t-test compared to control). (C) SA-βgal staining performed on replicatively senescent cells from adult and old mice was performed for the assessment of senescent cells (blue). Scale bars: 200µm. Changes in miR-24 levels affected the proportion of senescent cells, (n = 4-6, two-tailed unpaired Student's t-test compared to control). (D) Ki67 staining of primary myogenic progenitors demonstrates no difference in cell proliferation following miR-24 overexpression or inhibition. Scale bars: 200µm. n = 4-7; two-tailed unpaired Student t-Test. (E) The expression of senescence-associated genes was affected following miR-24 overexpression in myogenic progenitors from old mice. (n = 3-7, Kruskal-Wallis test followed by Dunn's multiple comparisons test with 95% Confidence Interval; expression relative to 18S is shown). All panels: Cells transfected with scrambled RNA were used as control group (Control); adult: 6-8 months old; old: 20-24 months old; p-value < 0.05 was considered as statistically significant (*p < .05; **p < .01; ***p < .001). Error bars show S.E.M.

Figure S4. miR-24 regulates differentiation, viability and senescence of human primary myogenic progenitors. Human primary myogenic progenitor cells isolated from adults were transfected with miR-24 or AM24. Cells transfected with the empty vector or scrambled control were used as control (Control). **(A)** MF 20 (anti-myosin heavy chain; green) and DAPI (blue) immunostaining were performed for myogenic differentiation and nuclei identification, respectively. **(B)** Viability assay was performed with ethidium bromide and acridine orange for the assessment of viable (green), apoptotic (yellow) and necrotic (red) cells. **(C)** SA-βgal staining was performed for the assessment of senescent cells (blue). Scale bars: 200μm. **(D)** Ki67 staining indicates no effects of miR-24 on human myoblast proliferation. miR-24 overexpression resulted in thinner myotubes, less viability and increased number of senescent cells (n = 3-4, two-tailed unpaired Student's t-test compared to Control group). p-value < 0.05 was considered as statistically significant (*p < .05; **p < .01; ***p < .001). Error bars show S.E.M.

Figure S5. Negative control for MitoSox Red staining and Mitosox Red staining in cells not treated or treated with H2O2 to test the working concentration od MitoSox Red.

Figure S6. Original western blot images.

Supplementary table 1: Age and gender of the patients donating a muscle biopsy for the isolation of human primary myogenic progenitor cells.

Donor ID	Age (years)	Gender	BMI
Donor 1	32	Female	<25
Donor 2	34	Female	<25
Donor 3	32	Female	<25
Donor 4	31	Female	<25
Donor 5	22	Female	<25
Donor 6	35	Female	<25

Supplementary table 2: Antibodies used for the isolation of satellite cells by FACS.

FACS Conjugated antibody	Company	Catalogue number	Dilution	FACS Aria filter
Anti-CD31-PE: PE Rat Anti-Mouse CD31. Clone: MEC 13.3. Isotype: Rat IgG2a, κ. 0.2 mg/ml	BD Biosciences PharmingenTM	561073	1:1333	575/26 (PE)
Anti-CD-45/PE: PE Rat Anti-Mouse CD45. Clone: 30-F11. Isotype: Rat IgG2b, κ. 0.2 mg/ml	(BD Biosciences PharmingenTM	553081	1:1333	575/26 (PE)
Anti-Sca1/FICT: FICT Rat Anti-mouse Ly-6A/E. Clone: E13-161.7. Isotype: Rat IgG2a, κ. 0.5 mg/ml	BD Biosciences PharmingenTM	553335	1:1333	530/30 (FITC)

Anti-Alpha 7 Integrin 647. Clone: R2F2. Isotype: Rat IgG2b. 1.0 mg/ml	AbLab	N/A	1:2000	660/20 (APC)
BV421 Rat Anti-Mouse CD34 Clone RAM34 (RUO). 0.2 mg/ml	BD Biosciences PharmingenTM	562608	1:1000	450/40 (Pacific Blue)
Fixable Viability Dye eFluor 780 (label dead cells)	Affimetrix eBiosciences	65-0865-14	1:4000	780/60 (APC-Cy7)

Supplementary table 3: List of primers, oligos and antibodies used for the study.

Gene	Company	Organism	Sequence (5'-3')/ Cat. Number
Hs_SNORD61_11 miScript Primer Assay	Qiagen	Human, mouse	MS00033705
Hs_miR-24_1 miScript Primer Assay	Qiagen	Human, mouse	MS00006552. Targets mature miR: UGGCUCAGUUCAGCAGGAACAG
Beta-actin Forward	Sigma-Aldrich	Mouse	GATCAAGATCATTGCTCCTCCTG
Beta-actin Reverse	Sigma-Aldrich	Mouse	AGGGTGTAAAACGCAGCTCA
18S rRNA Forward	Sigma-Aldrich	Mouse	CGGCTACCACATCCAAGGAAGG
18S rRNA Reverse	Sigma-Aldrich	Mouse	CCCGCTCCCAAGATCCAACTAC
Beta-2 microglobulin Forward	Sigma-Aldrich	Mouse	GGAGAATGGGAAGCCGAACA
Beta-2 microglobulin Reverse	Sigma-Aldrich	Mouse	TCTCGATCCCAGTAGACGGT
p16 Forward	Sigma-Aldrich	Mouse	TGGTCACTGTGAGGATTCAGC
p16 Reverse	Sigma-Aldrich	Mouse	GTTGCCCATCATCATCACCTGG
p21 Forward	Sigma-Aldrich	Mouse	ATCCAGACATTCAGAGCCACAG
p21 Reverse	Sigma-Aldrich	Mouse	TCGGACATCACCAGGATTGG
Prdx6 Forward	Sigma-Aldrich	Mouse/ Human	TTGATGATAAGGGCAGGGAC
Prdx6 Reverse	Sigma-Aldrich	Mouse/ Human	CTACCATCACGCTCTCTCCC
Tumor protein p53 Forward	Sigma-Aldrich	Mouse	CACGTACTCTCCTCCCCTCAAT
Tumor protein p53 Reverse	Sigma-Aldrich	Mouse	AACTGCACAGGGCACGTCTT
mPRDX6-202 5'UTR Forward	Sigma-Aldrich	Mouse	GCCCCGCCCACTCGGCCAGC
mPrdx6-202 5'UTR Reverse	Sigma-Aldrich	Mouse	AGCAACCCTCCGGGCATGGC
mPrdx6-202 5'UTR WT	Sigma-Aldrich	Mouse	GCCCCGCCCACTCGGCCAGCACTGATCTAGGTCTCCGCAG**GAGCC**CGCCCGCTGCTCACTGCTGCGGCTGCGCCTCCTTGTTCTCAGCGTCACCACTGCCGCCATGCCCGGAGGGTTGCT

mPrdx6-202 5'UTR 24 MUT	Sigma-Aldrich	Mouse	GCCCCGCCCACTCGGCCAGCACTGATCTAGGTCTCCGCAG**GATCC**CGCCCGCTGCTCACTGCTGCGGCTGCGCCTCCTTGTTCTCAGCGTCACCACTGCCGCCATGCCCGGAGGGTTGCT
Bcl-2 Forward	Sigma-Aldrich	Mouse	CTGCAAATGCTGGACTGAAA
Bcl-2 Reverse	Sigma-Aldrich	Mouse	TCAGGAGGGTTTCCAGATTG
H2ax Forward	Sigma-Aldrich	Mouse	GGCCTGTGGACAAGAGTTCTAT
H2ax Reverse	Sigma-Aldrich	Mouse	GCCCATTAAATCTCCCCACT
P53 Forward	Sigma-Aldrich	Mouse	CACGTACTCTCCTCCCCTCAAT
P53 Reverse	Sigma-Aldrich	Mouse	AACTGCACAGGGCACGTCTT
Bcl-2 Forward	Sigma-Aldrich	Human	TCGCCCTGTGGATGACTGA
Bcl-2 Reverse	Sigma-Aldrich	Human	CAGAGACAGCCAGGAGAAATCA
H2ax Forward	Sigma-Aldrich	Human	CATGTCGGGCCGCGGCAA
H2ax Reverse	Sigma-Aldrich	Human	GTGGCGCTGGTCTTCTTG
P16 Forward	Sigma-Aldrich	Human	GAAGGTCCCTCAGACATCCCC
P16 Reverse	Sigma-Aldrich	Human	CCCTGTAGGACCTTCGGTGAC
P21 Forward	Sigma-Aldrich	Human	GGCAGACCAGCATGACAGATTTC
P21 Reverse	Sigma-Aldrich	Human	CGGATTAGGGCTTCCTCTTGG
S29 Forward	Sigma-Aldrich	Mouse/ Human	ATGGTCACCAGCAGCTCTA
S29 Reverse	Sigma-Aldrich	Mouse/ Human	GTATTTGCGGATCAGACCGCT

Supplementary table 4: Table of reagents used for the experiments.

Product	Company	Catalogue number
Barium chloride	Sigma-Aldrich	202738
AllStars Negative Control siRNA Print	Qiagen	1027280 (discontinued)
miRIDIAN Scr control	Dharmacon	IN-122262-00-70
Syn-mmu-miR-24-3p	Qiagen	MSY0000219
Anti-mmu-miR-24-3p	Qiagen	MIN0000219 (discontinued)
Anti-miR-24-3p	Dharmacon	IH-122261-00-70
Mouse Prdx6 siRNA	ThermoFisher Scientific	s62375 (discontinued), s62376

Human Prdx6 siRNA	ThermoFisher Scientific	s18428 s18429
Lipofectamine 2000	ThermoFisher Scientific	11668019
MF20 primary antibody. Antigen: myosin, sarcomere (MHC). 211 ug/ml	Developmental Studies Hybridoma Bank	MF20-c 2ea
Rabbit mAb to Ki67 [SP6].	Abcam	ab16667
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate.	Invitrogen	A-11029
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate.	ThermoFisher Scientific	A-11034
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Sigma-Aldrich	D9542
Senescence β-Galactosidase Staining Kit Cell Event Cell senescence kit	Cell Signaling Technology Thermo Fisher	9860 C10850
Acridine Orange hydrochloride solution, 10 mg/mL in H_2O	Sigma-Aldrich	A8097
Ethidium bromide solution. BioReagent, for molecular biology, 10 mg/mL in H_2O	Sigma-Aldrich	E1510
Methanol	Fisher	M/4000/PC17
PBS (immunostaining)	Sigma-Aldrich	P4417
Tween-20	Sigma-Aldrich	P1379
Wheat Germ Agglutinin (WGA), Fluorescein	Vector Laboratories	FL-1021
Fluoromount	ThermoFisher	00-4958-02
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride). 1mg/ml	Sigma-Aldrich	D9542
miRNeasy Mini Kit	Qiagen	217004
TRIzol Reagent	Life Technologies	15596-018
Chloroform:Isoamyl alcohol 24:1	Sigma-Aldrich	C0549
Isopropanol	Sigma-Aldrich	I9516
RNAse-free water	Sigma-Aldrich	3098
Sodium acetate	Sigma-Aldrich	S2889
Nanodrop 2000	ThermoFisher Scientific	N/A
Superscript II Reverse Transcriptase	Life Technologies	18064
Random Hexamers (50 μM)	ThermoFisher	N8080127
25X dNTP Mix (100 mM)	ThermoFisher	4368814
RiboLock RNase Inhibitor (40 U/μL)	ThermoFisher	EO0381

miRScript RT II	Qiagen	218161
miRScript SybrGreen PCR Kit	Qiagen	218073
T100 Thermal Cycler	Bio-Rad	1861096
CFX Connect Real-Time PCR Detection System	Bio-Rad	1855201
RNU-6 qPCR primer	Qiagen	MS00033740
Snord-61 qPCR primer	Qiagen	MS00033705
miR-24_1 miScript Primer Assay	Qiagen	MS00006552
Select agar	Sigma-Aldrich	A5054
MyTaq Red Mix	Bioline	BIO-25043
GeneJET Genomic DNA Purification Kit	Thermo Scientific	K0721
One Shot TOP10 Chemically Competent E. coli	Invitrogen	C404010
SYBR Safe DNA Gel Stain	Invitrogen	S33102
DNA Gel Loading Dye (6X)	Thermo Scientific	R0611
UltraPure Agarose	Invitrogen	16500500
GFP Tag Antibody, ABfinity Rabbit Monoclonal	ThermoFisher Scientific	G10362
CM-H2DCFDA (General Oxidative Stress Indicator)	Invitrogen	C6827
FLUOstar OPTIMA microplate reader	BMG Labtech	N/A
Hydrogen peroxide solution 30 % (w/w) in H2O, contains stabilizer	Sigma-Aldrich	H1009
1x RBC (Red Blood Cell) Lysis Buffer	eBioscience	00-4333-57
FACS Aria III Flow Cytometer	BD Biosciences	N/A
MitoSox Red	Thermo Fisher	M36008
MitotTracker Red CM-H2Xros	Thermo Fisher	M7513
HCS DNA damage kit	Thermo Fisher	H10292
C1 confocal laser scanning microscope system. 10x magnification.	Nikon	N/A
Axiovert 200 inverted microscope. 10x magnification.	Carl Zeiss	N/A
EVOS M5000	Thermo Fisher	N/A
EVOS M7000	Thermo Fisher	N/A
SOD1	Abcam	
HSP70	Abcam	Ab181606