# Integrative stress response activation in aged label retaining MuSCs improves regeneration

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**INTRODUCTION**: For efficient regeneration, muscle stem cells (MuSCs) transition out of quiescence through a series of activated states and this pool of MuSCs are highly heterogenous. Label retaining cells (LRCs) have higher potency and activate rapidly, non-label retaining cells (nLRCs) are more numerous in aged and activate more slowly (Chakkalakal et al 2021). During aging, MuSCs lose their resiliency and undergo apoptosis at a higher rate compared to adult, particularly within LRCs. The integrative stress response (ISR) pathway governs the ability of a cell to adapt to stress through blocking global protein synthesis and selectively translating genes to protect that cell through the transcription factor *Activating transcription factor 4 (Atf4)*. Therefore, we hypothesize that enforcing the ISR in aged MuSCs will protect LRCs and accelerate regeneration through activation.

**METHODS**: All mice were males (IACUC approved) because our question/aim wanted to exclude hormonal fluctuations (estrogen) as a confounding variable, which is known to alter MuSC function in mice (Seko et al 2020). Adult and Aged 22-month C57BL/6J mice muscle fibers were isolated and cultured over 24h in the absence or presence of 10 μM Sal003 an ISR activator (ISR-a), fixed and stained for MuSCs (pax7) and the ISR regulator peIF2α. Aged single fibers were also chased with Edu (marker of activation) with ISR-a and 200 nM ISRIB an ISR inhibitor (ISR-i). Aged MuSCs were isolated via Fluorescence-Activated Cell Sorting and cultured over 48h and assessed for apoptosis (Caspase-3). Using shRNA lentivirus, *Atf4* was deleted in aged MuSCs in the absence and presence of ISR-a and assessed for activation (Edu) over 48h of culture. TetO-H2B-GFP mice were embryonically labelled with doxycycline and chased until 22 months (to assess LRCs and nLRCs). MuSCs were cultured in the ISR-a and ISR-i and assessed for activation, apoptosis. Cells were also cultured in ISR-a for 2 hours and transplanted into barium chloride (BaCl) injured muscle and after 30 days muscle was assessed for $GFP^+$ cells. After BaCl injury in aged mice, ISR-a (1mg/kg) was injected via intraperitoneal injection (IP) and cross-sectional area (CSA) of centrally nucleated fibers was assessed after 21 days.

**RESULTS**: Aged peIF2α was suppressed and did not increase during MuSC activation, unlike adult MuSCs (Fig 1A). ISR-a stimulated an ISR response and improved MuSC activation (Fig 1B) whereas ISR-i slowed activation and increased apoptosis (Fig 1C). Increased aged MuSC activation with ISR-a acted through *Atf4* (Fig 1D). ISR-a increased the activation of nLRCs compared to LRCs, with ISR-i reducing both subpopulations (Fig 2A). This supported a trend to increased cell survival with ISR-a in nLRCs and increased apoptosis in all MuSCs with ISR-i (Fig 2B). However, transplanted MuSCs with ISR-a increased the amount of LRCs with no change in nLRCs (Fig 3A-B). After injury, ISR-a improved CSA of nucleated fibers in aged compared to control (Fig 3C-D).

**DISCUSSION**: Muscle regeneration is delayed with age, which is partly due to abrogated MuSC activation and impaired resilience. The ISR is suppressed basally in aged MuSCs and does not increase in response to injury compared to adult. This is altered by evoking peIF2α through an ISR-a which causes accelerated activation through the downstream regulator *Atf4*. The effects of ISR-a on MuSCs subpopulations are context dependent, *in vitro* activation of nLRCs is improved, whilst via *in vivo* transplants during regeneration caused an increase in LRCs. Importantly, ISR-a improved overall aged regeneration, potentially through increased activation and resiliency.

**SIGNIFICANCE**: Aged individuals have greater risk of muscular injury and impaired regeneration increases risk of re-injury, causing increased socioeconomical burden on healthcare systems. Here we demonstrate aged regeneration can be improved through therapeutically modulating the ISR, with clinical trial potential.

**REFERENCES**: Chakkalakal, J.V., Jones, K.M., Basson, M.A. and Brack, A.S., 2012. The aged niche disrupts muscle stem cell quiescence. Nature, 490(7420), pp.355-360. Seko, D., Fujita, R., Kitajima, Y., Nakamura, K., Imai, Y. and Ono, Y., 2020. Estrogen receptor β controls muscle growth and regeneration in young female mice. Stem Cell Reports, 15(3), pp.577-586.

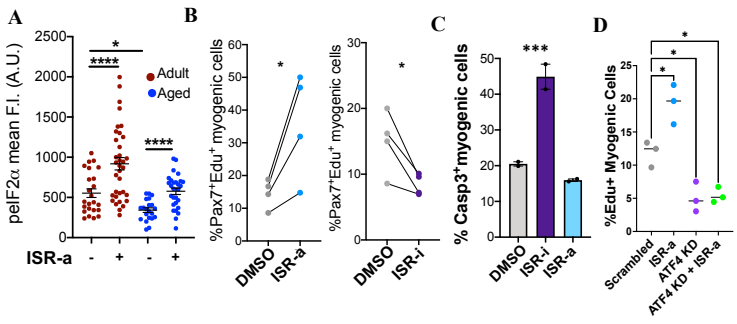
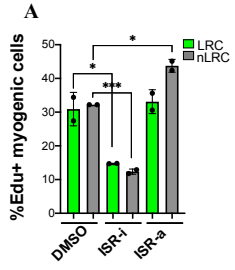



**Fig1.A-C.** ISR-a and ISR-i on MuSC peIF2α intensity, percent $Pax7^+Edu^+$ cells and $Caspase3^+$ cells in culture. **D.** Percent $Edu^+$ myogenic cells after *Atf4* shRNA deletion. Mean±SEM, N=2/3. *p<0.05, ***p<0.001, ****p<0.0001.



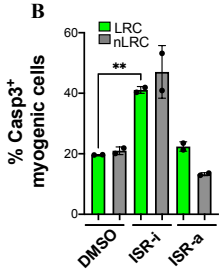
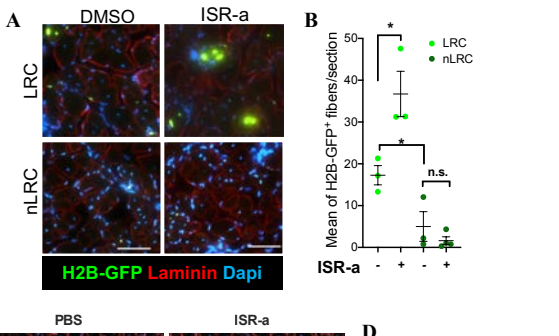




**Fig2.A-B.** Percent $Edu^+$ aged myogenic cells in LRCs and nLRCs with ISR-a and ISR-b. **B.** $Caspase^+$ aged myogenic cells in LRCs and nLRCs with ISR-a and ISR-b. Mean±SEM, N=2. *p<0.05, **p<0.01,***p<0.001.



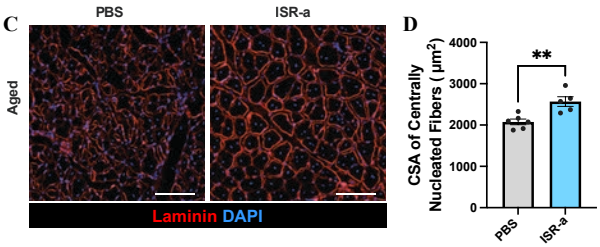




**Fig3. A-B.** Representative images and quantification 30 days after transplanted aged H2B-GFP LRCs and nLRCs into pre-injured muscle. **C-D.** Representative images and CSA quantification 21days after ISR-a IP injections following muscle injury in aged mice. Mean±SEM, N=3, 5/6. *P<0.05, **p<0.01.