Cell Biology

Rejuvenating aged osteoprogenitors for bone repair

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Aging reduces tissue regeneration capacity, posing challenges for an aging population. In this **fundamental** study, Reeves et al. show that by combining Wnt-mediated osteoprogenitor expansion (using a special bandage) with intermittent fasting, calvarial bone healing can be restored in aged animals. Intermitted fasting improves osteoprogenitor function by rescuing aging-related mitochondrial dysfunction, which can also be achieved by nicotinamide mononucleotide (NMN) supplementation or by modulating the gut microbiome. By employing rigorous histological, transcriptomic, and imaging analyses in a clinically relevant model, the authors provide **compelling** evidence supporting the conclusions. The therapeutic approach presented in this study shows promise for rejuvenating tissue repair, not only in bones but potentially across other tissues.

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Abstract

Aging is marked by a decline in tissue regeneration, posing significant challenges to an increasingly older population. Here, we investigate age-related impairments in calvarial bone healing and introduce a novel two-part rejuvenation strategy to restore youthful repair. We demonstrate that aging negatively impacts the calvarial bone structure and its osteogenic tissues, diminishing osteoprogenitor number and function and severely impairing bone formation. Notably, increasing osteogenic cell numbers locally fails to rescue repair in aged mice, identifying the presence of intrinsic cellular deficits. Our strategy combines Wnt-mediated osteoprogenitor expansion with intermittent fasting, which leads to a striking restoration of youthful levels of bone healing. We find that intermittent fasting improves osteoprogenitor function, benefits that can be recapitulated by modulating NAD^+ dependent pathways or the gut microbiota, underscoring the multifaceted nature of this intervention. Mechanistically, we identify mitochondrial dysfunction as a key component in age-related decline in osteoprogenitor function and show that both cyclical nutrient deprivation and Nicotinamide mononucleotide rejuvenate mitochondrial health, enhancing osteogenesis.

These findings offer a promising therapeutic avenue for restoring youthful bone repair in aged individuals, with potential implications for rejuvenating other tissues.

Introduction

Flat bones like the sternum, scapula, pelvic bones, and skull bones play crucial roles in the body, including protecting organs and anchoring muscles. Their thin structure makes them prone to fractures(Huelke and Compton, 1983), leading to pain, disability, and complications like internal bleeding or organ damage(Ren et al., 2020). As individuals age, flat bones become more brittle, increasing the risk of fractures(Beedham et al., 2019). Skull fractures, common in head injuries at any age(Carson, 2009), carry a higher mortality rate even when considering trauma severity(Tsai et al., 2022). Aging affects the shape and fragility of calvarial bones(Cotofana et al., 2018)(Torimitsu et al., 2014), but the impact on osteogenic tissues supporting bone repair, such as the calvarial periosteum and suture mesenchyme remains elusive.

In the calvaria, bones form and repair through intramembranous ossification(van den Bos et al., 2008). Osteoblasts which deposit pre-mineralized bone tissue(Bianco and Robey, 2015) arise from $CD90^+$ osteoprogenitor populations(Chan et al., 2015, 2013; Kim et al., 2016), which are derived from heterogeneous populations of skeletal stem cells (SSCs)(Chan et al., 2015, 2013; Kim et al., 2016). Osteoprogenitors reside in soft tissues alongside endothelial and other cell types that interface with mineralized bone tissue. The two main described osteoprogenitor niches are the periosteum, which overlays all flat bones(Debnath et al., 2018), and the suture mesenchyme, only present in the skull, between calvarial bones(Wilk et al., 2017). Localized Wnt ligands (Wnts) play crucial roles in regulating osteogenesis, influencing stem cells, progenitors, and mature osteoblasts(Garcin and Habib, 2017; Liu et al., 2009; Lowndes et al., 2016; Matsushita et al., 2020; Okuchi et al., 2021). We recently showed that the Wnt3a-bandage, a transplantable polymer that harbors covalently immobilized Wnt3a proteins, combined with an engineered human osteogenic tissue, can promote bone repair in adult immunodeficient mice(Okuchi et al., 2021).

In this study, using a clinically relevant immunocompetent mouse model, we examined how aspects of the periosteum and suture mesenchyme change with age, how they respond to injury, and the influence of localized Wnts throughout aging.

Young mice achieve almost complete bone repair of calvarial defects considered ‘critical sized’ in adults, which have limited self-healing ability. We found that age-related changes in bone tissue begin to manifest during adulthood, including increased bone porosity, altered cellular mechanical cues, and declines in mitochondrial activity, vascularity, and the proportion and function of $CD90^+$ osteoprogenitor cells within the periosteum and suture mesenchyme. While Wnt bandage increases $CD90^+$cells at the defect site regardless of age, this increase only sufficiently promotes repair comparable to young animals in adult mice, not aged mice. Our study characterized a more pronounced decline in osteogenic compartments in aged mice, encompassing reduced $CD90^+$ cell proportion, function, and mitochondrial bioenergetics, alongside other contributing factors. This multifaceted decline leads to severe age-related bone repair deficiency, only partially alleviated by the Wnt3a-bandage, underscoring the need for combinatorial approaches.

To counteract age-related decline in bone repair, we combined localized Wnt3a stimulation with systemic metabolic interventions. In aged mice with critical-size defects, alternating-day intermittent fasting restored osteogenic compartment health, improving $CD90^+$ cell function as well as bone remodelling and significantly enhanced bone repair to levels comparable to younger animals. This effect was recapitulated by even shorter-term supplementation of the gut bacteria *Akkermansia muciniphila* or nicotinamide mononucleotide (NMN – an NAD^+ precursor) in aged

mice, highlighting the multifaceted mechanisms of action of intermittent fasting. We further identify mitochondrial dysfunction as a key component in age-related decline in osteoprogenitor function and show that both cyclical nutrient deprivation or NMN rejuvenate mitochondrial health and enhance osteogenesis *in vitro*.

In summary, our findings highlight that aging tissues retain the potential for health improvements through interventions. Our data reveals an intriguing avenue of research where metabolic and microbiome interventions can be repurposed to treat tissue damage in aged repair-deficient individuals.

Results

Parietal bone structure deteriorates with aging

Initially, we analyzed the changes that occur in calvarial bone of female C57BL6/J mice during aging at the macro-structure. Female mice were selected for this study due to their distinct characteristics, such as delayed healing and higher fracture risk (Haffner-Luntzer et al., 2021; Ortona et al., 2023), which provide a more challenging model for evaluating bone repair strategies. Young mice (6 weeks) are within the period of fastest bone formation and growth, Adult mice (13 weeks) at healthy homeostasis, and Aged mice (> 88 weeks) correspond to declining skeletal health as a model of >70 year old humans(Dutta and Sengupta, 2016). We evaluated the calvaria of these animals using micro-computed tomography (microCT). The proportion of the total bone volume taken up by mineralized bone tissue (Bone Volume/Total Volume, BV/TV) significantly decreased with age (**Fig. 1a-b**). Cortical bone mineral density (Ct.BMD), the measure of the density of the mineralized bone tissue alone, showed that Aged mice have similar mineral density to Young and Adult mice (nonsignificant differences). The overall thickness from outer surface to inner surface of the parietal bone significantly increased with age (**Figure 1 – figure supplement 1a-d**).

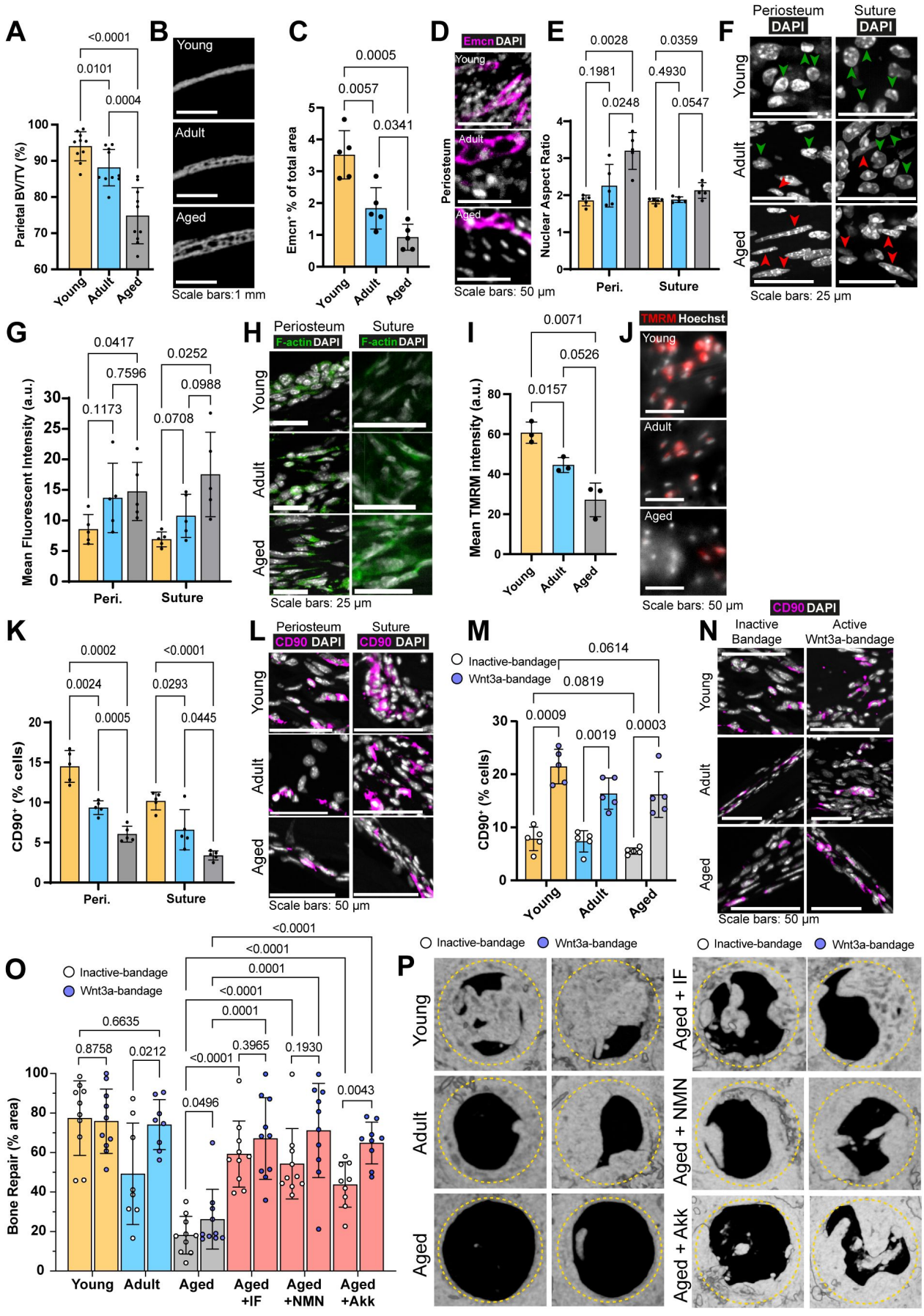
A
Parietal BV/TV (%)
<0.0001
0.0101
0.0004
Young
Adult
Aged
B
Young
Adult
Aged
Scale bars:1 mm
C
Emcn⁺ % of total area
0.0005
0.0057
0.0341
D
Emcn DAPI
Periosteum
Scale bars: 50 µm
E
Nuclear Aspect Ratio
0.0028
0.0359
0.1981
0.4930
0.0248
0.0547
Peri.
Suture
F
Periosteum
Suture
DAPI
Scale bars: 25 µm
G
Mean Fluorescent Intensity (a.u.)
0.0417
0.0252
0.7596
0.0988
0.1173
0.0708
H
F-actin DAPI
Scale bars: 25 µm
I
Mean TMRM intensity (a.u.)
0.0071
0.0526
0.0157
J
TMRM Hoechst
Scale bars: 50 µm
K
CD90⁺ (% cells)
0.0002
<0.0001
0.0024
0.0293
0.0005
0.0445
L
CD90 DAPI
Scale bars: 50 µm
M
Inactive-bandage
Wnt3a-bandage
0.0614
0.0819
0.0009
0.0019
0.0003
N
Inactive Bandage
Active Wnt3a-bandage
Scale bars: 50 µm
O
Bone Repair (% area)
<0.0001
<0.0001
0.0001
<0.0001
0.0001
0.6635
0.8758
0.0212
0.3965
0.1930
<0.0001
0.0496
0.0043
Young
Adult
Aged
Aged +IF
Aged +NMN
Aged +Akk
P
Aged + IF
Aged + NMN
Aged + Akk

Figure 1.

Changes to the calvarial bone, periosteum and suture mesenchyme, and bone repair with aging and interventions.

(**A**) Parietal Bone Volume/Total Volume (BV/TV) and (**B**) representative transverse micro computed tomography (microCT) sections in Young, Adult, and Aged mice. Statistical analysis: Welch's test in n = 10 mice per condition. (**C**) Mean cross sectional area taken up by Endomucin (Emcn)-positive vascular cells and (**D**) representative immunofluorescence staining of Emcn (magenta) and DAPI (grey) in Young, Adult, and Aged mouse periosteum. Statistical analysis: Welch's tests in n = 5 mice per condition. (**E**) Nuclear aspect ratio (long axis divided by short axis) of cells within the periosteum and suture mesenchyme and (**F**) representative DAPI-stained nuclei in Young, Adult, and Aged mice. Statistical analysis: Welch's tests in n = 5 mice per condition. Green and red arrows highlight rounded and elongated nuclei, respectively. (**G**) Mean Phalloidin-488 fluorescence intensity and (**H**) representative images for filamentous actin (F-actin, green) and DAPI (grey) in Young, Adult, and Aged mouse periosteum and suture mesenchyme. Statistical analysis: Welch's tests in n = 5 mice per condition. (**I**) Mean intensity and (**J**) representative images of live TMRM staining (red) and Hoechst (grey) in Young, Adult, and Aged mouse periosteum *ex vivo*. Statistical analysis: Welch's tests in tissue from n = 3 mice per condition. (**K**) Percentage of $CD90^+$ cells within the periosteum and suture mesenchyme, expressed relative to the total cellular number ($DAPI^+$) in Young, Adult, and Aged mice. Representative images (**L**) show CD90 immunostaining (magenta) and DAPI (grey). Statistical analysis: Welch's tests in n = 5 mice per condition. (**M**) Percentage of $CD90^+$ cells within calvarial defects treated with Wnt3a-bandages (blue points) or inactive controls (white points), expressed relative to the total cellular number ($DAPI^+$) in Young, Adult, and Aged mice. (**N**) Representative images show CD90 immunostaining (magenta) and DAPI (grey). Statistical analysis: ratio-paired two-tailed t tests in n = 5 mice per condition. (**O**) Percentage of repair and (**P**) representative top-down microCT images within calvarial defects treated with Wnt3a-bandages (blue points) or inactive controls (white points). Statistical analysis within age groups: ratio-paired two-tailed t tests; between age groups and Aged mice following the IF protocol or NMN or Akk supplementation: Welch's tests; Young, Aged, Aged + IF, Aged + NMN, n = 10 mice; Aged + Akk, n = 9; Adult, n = 8. Complete statistical analysis between aged groups and treatments can be found in Supplementary File 1. The yellow dashed circle highlights the 2 mm diameter defect size.

As parietal bone formation and maintenance is regulated by the cells within the periosteum and suture mesenchyme(Debnath et al., 2018; Li et al., 2021; Maruyama et al., 2016), we next defined the changes that occur within these compartments during aging.

Age-related changes in vascularization, nuclear morphology, F-actin levels and osteoprogenitor proportions in osteogenic compartments

Blood vessels in the calvaria bone support tissue survival by delivering oxygen and nutrients, regulating bone metabolism, and contributing to its maintenance and remodeling, while tissue stiffness influences cell behavior and osteoprogenitors differentiate into cells essential for these processes. Therefore, we aimed to characterize blood vessels, nuclear and cytoskeletal morphology, and osteoprogenitor proportions within the periosteum and suture mesenchyme. Initially, we established that 97% endothelial cells in the periosteum and the suture mesenchyme (osteogenic compartments) express Endomucin and CD31 (**Figure 1 – figure supplement 1e, f**). We found that the proportion of Endomucin-positive ($Emcn^+$) blood vessels in the periosteum significantly decreases with age (**Fig. 1c-d**, **Figure 1 – figure supplement 1g-j**). Further vascular histomorphometry shows that, in the periosteum, blood vessel lumen area is consistent throughout aging, indicating that it is the decrease in the blood vessel number per area of tissue that drives the declining blood vessel density. In addition, we find that the thickness of the periosteum itself also decreases rapidly with age (**Figure 1 – figure supplement 1k, l**). Taking increased periosteum thickness and density of blood vessels into account, the estimated blood

supply (vessel density multiplied by periosteum thickness) of the whole Young periosteum is over 12-fold higher than that of Aged periosteum. In the suture mesenchyme, blood vessels are larger than in the periosteum, and overall, the vascularization remains stable throughout aging (**Figure 1 – figure supplement 2a, b**).

Vascular phenotypes can be influenced by tissue mechanics (Xie et al., 2018). Therefore, we aimed to measure mechanically-driven aspects of cellular morphology in periosteal and suture mesenchymal cells during aging. Nuclear shape is a product of intracellular(Dahl et al., 2008) and extracellular factors, such as the stiffness of the surrounding matrix(Lovett et al., 2013). Morphologically and confirmed by the mean Nuclear Aspect Ratio (NAR, long axis length divided by short axis length) we found significant elongation of nuclei in Aged mice periosteum and to a lesser extent, in the suture mesenchyme (**Fig. 1e-f**). Lamin B1, a key nuclear lamina protein involved in regulating nuclear function and organization, showed no significant changes across age groups(bin Imtiaz et al., 2021; Kim, 2023; Tran et al., 2016) (**Figure 1 – figure supplement 2i-j**)

Nuclear morphology is frequently interlinked with actin cytoskeleton organization, influencing cell migration, proliferation(Ambriz et al., 2018) and the response to mechanical force imparted on them through the extracellular matrix(Fletcher and Mullins, 2010; Stricker et al., 2010). We quantified filamentous actin (F-actin) using fluorescent-Phalloidin staining in periosteum and suture mesenchyme (**Fig. 1g-h**, **Figure 1 – figure supplement 2h**). Aging led to a progressive increase of F-actin, peaking at a 2.5-fold rise between young and Aged mice in suture mesenchyme.

Beyond morphological and cytoskeletal changes, we questioned whether aging periosteal cells experience changes to their cellular energy supply. Adenosine triphosphate (ATP) generation, crucial for cell function, relies largely on mitochondrial membrane potential(Zorova et al., 2018). This can be measured by determining the intensity of Tetramethylrhodamine, methyl ester (TMRM), which accumulates in active mitochondria with intact membrane potentials in live cells(Creed and McKenzie, 2019). *Ex vivo* TMRM intensity was significantly higher in freshly-harvested samples of Young mouse periosteum, than in Adult and Aged mice (**Fig. 1i-j**). This finding is unlikely to be an artefact of differential ECM densities interfering with diffusion as performing the same experiment in explanted cells (i.e. without ECM, **Figure 1 – figure supplement 2c, d**) gave a similar trend of results. We also confirmed equal Hoechst diffusion and staining throughout the *ex vivo* tissue experiment (**Figure 1 – figure supplement 2e, f**).

Given these age-related differences in the osteogenic compartments, we aimed to elucidate changes to the $CD90^+$ osteoprogenitors (Chan et al., 2015; Kim et al., 2016), (**Figure 1 – figure supplement 2g**). Using in situ immunofluorescence, we found that the proportion of $CD90^+$ in the periosteum and the suture mesenchyme significantly decreases with age, with only around one third retained in Aged mice in each compartment (**Fig. 1k-l**).

These tissue and cell-level changes indicate a significant and multifaceted age-related decline in the calvarial osteogenic compartments. The periosteum and the suture mesenchyme also serve as the primary sources of cells that contribute to osteogenesis during calvarial bone repair(Jeffery et al., 2022; Wilk et al., 2017). Hence, we next characterized how calvarial bone repair is affected by age and how the nearby cells respond to the Wnt3a-bandage.

Wnt3a-bandages increase $CD90^+$ cells in repair areas of Aged and Adult mice and enhance bone repair

Critical-sized calvarial bone defects, challenging to heal without intervention, serve as models to quantitatively evaluate bone repair. Our prior work demonstrated that applying a Wnt3a-bandage and cellular osteogenic construct to the defect site significantly enhances bone repair in adult

immunocompromised mice(Okuchi et al., 2021). Considering the crucial role of the immune system in bone healing(Loi et al., 2016) and the prevalence of bone fractures in immunocompetent individuals, we assessed bone repair in female wildtype (C57BL6/J) mice of varying ages. Mice underwent bilateral critical-sized (2 mm) full-thickness calvarial defect surgery in the parietal bones and were treated with one active Wnt3a-bandage and one chemically-inactivated Wnt3a control bandage. Wnt3a-bandage activity was confirmed *in vitro* (**Figure 1 – figure supplement 3a-c**) before transplantation.

We assessed bone repair by measuring the area percentage of re-mineralized tissue within the defect site using microCT after 10 weeks. Interestingly, Young mice exhibited robust bone healing even without intervention, highlighting the natural regenerative capacity of bone in young mice. (**Fig. 1o-p**, Supplementary File 1). In adult mice, Wnt3a-bandage treatment significantly enhanced bone repair compared to the control bandage, achieving levels comparable to those observed in young mice. Aged mice exhibited severely impaired repair with both bandages, though Wnt3a-bandage showed a modest but significant increase. New bone formed under Wnt3a-bandage displayed improved lamellar structure (striations) akin to mature undamaged bone (**Figure 1 – figure supplement 3d**).

To uncover cellular mechanisms behind the improved healing, we examined $CD90^+$ osteoprogenitor cells in the defect site. The proportion of $CD90^+$ cells was significantly increased by the Wnt3a-bandage across age groups, compared to control bandages (**Fig. 1m-n**). Wnt3a-bandages locally increase $CD90^+$ cells, but despite similar numbers across ages, the effect of the Wnt3a-bandage on bone repair in Aged mice was limited. We hypothesized, therefore, that the functionality of $CD90^+$ cells, rather than their number alone, may drive age-related differences in bone repair outcomes.

To investigate this, we initially examined the influence of the Wnt-bandage on $CD90^+$ cells across various age groups in terms of Wnt/β-catenin signaling. The proportion of nuclear β-catenin (**Figure 1 – figure supplement 4a-c**), indicative of Wnt/β-catenin signaling activation(Archbold et al., 2012), was significantly increased in $CD90^+$ cells near the active Wnt3a-bandage compared to the inactive bandage. Importantly, the proportion of nuclear β-catenin remained similar regardless of age, suggesting the Aged $CD90^+$ have the capacity to activate the pathway similar to younger $CD90^+$ cells.

We then explored the effects of the Wnt3a-bandage on $CD90^+$ cell proliferation and migration near the bandage. Our results demonstrate that the proportion of $CD90^+$ stained with the proliferation marker Ki67 or MMP2, a Type IV collagenase enzyme linked to migration(Ries et al., 2007) and ECM remodeling in flat bones (Egeblad et al., 2007; Mosig et al., 2007; Zhong-Sheng et al., 2022), appeared increased in $CD90^+$ near Wnt3a-bandage. While this increase was not statistically significant compared to inactive control bandage groups in Aged mice, it was statistically significant in Adult mice (**Figure 1 – figure supplement 4b-e**).

Considering that $CD90^+$ cells inside the defect are ultimately derived from cells that are harbored in the nearby periosteum and suture mesenchyme, we next analyzed the transcriptomes of $CD90^+$ to glean insights into their functional differences during aging.

Transcriptional shifts occur in calvarial $CD90^+$ cells with aging

Single-cell RNA sequencing (scRNAseq) was performed on live cells immediately following dissection and tissue digestion from the periosteum and suture mesenchyme in Young, Adult, and Aged mice, with data processing to deplete immune ($CD45^+$) cells. $CD90^+$ subpopulations of cells (cluster 0, 1 and 3 in the periosteum; cluster 10 in the suture mesenchyme) were identified within both tissues (**Figure 2 – figure supplement 1a-h**), with high transcriptomic similarity to cells expressing Prrx1,Cathepsin K (Ctsk) and Ddr2(Bok et al., 2023; Debnath et al., 2018) (**Figure 2**

– figure supplement 2), markers of periosteal and suture SSCs(Duchamp de Lageneste et al., 2018; Wilk et al., 2017). We also found that the CD90$^+$ population includes minor subsets of cells that express other SSC/progenitor markers including CD200/CD51 (Cd200/Itgav)(Menon et al., 2021), Gli1(Zhao et al., 2015), Axin2(Maruyama et al., 2016) and α-SMA (Acta2)(Ortinau et al., 2019). Therefore, based on functional studies(Kim et al., 2016; Nusspaumer et al., 2017) and our transcriptomic analysis, the CD90$^+$ population encompasses a heterogeneous group of cells that are capable of osteogenic differentiation. Next, we will describe selected transcriptomic changes in CD90$^+$ cells during aging. For Gene ontology number, comparisons and statistics, please see Supplementary File 2.

Comparing Young CD90$^+$ cells with Aged CD90$^+$ cells in the periosteum (**Figure 2 – figure supplement 3a**), 542 genes were significantly upregulated. Of these, 38 corresponded to Gene Ontology (GO) term 'Cytosolic ribosome', linked to increased 'Collagen-containing extracellular matrix'. Similarly in Young vs Aged suture CD90$^+$ cells (**Figure 2 – figure supplement 3b**), 879 significantly upregulated genes are enriched for 'Structural constituent of ribosome' and 'Extracellular matrix structural constituent'. Young mice at 6 weeks of age undergo significant skeletal growth(Jilka, 2013), hence this data closely corroborates the increase in collagenous matrix deposition (both soft and bone tissue) observed in histological sections of bone defects in Young mice.

Given the effect of the Wnt3a-bandage on the Aged bone repair environment, we next probed the differentially expressed genes for components of the Wnt/β-catenin pathway. The transcript's expression of Wnt2 ligand, often implicated in the Wnt/β-catenin pathway(Goss et al., 2009; Jung et al., 2015), was significantly downregulated with age in suture CD90$^+$ cells (**Figure 2 – figure supplement 3b,d**). Further, Wnt co-receptor Lrp6 expression significantly declined with age in periosteal CD90$^+$ cells (**Figure 2 – figure supplement 3a**). Collectively, this suggests compromised Wnt signaling in the periosteum and suture mesenchyme during aging.

At the Adult and Aged timepoints, bone is at healthy and declining homeostasis, respectively. We compared Adult and Aged CD90$^+$ cells in the periosteum (**Figure 2 – figure supplement 3c**). Here, the 637 genes significantly upregulated in Adult vs Aged were vastly enriched for GO terms 'NADH dehydrogenase complex assembly', 'NADH dehydrogenase (ubiquinone) activity' and 'Oxidative phosphorylation'. Likewise, in the suture mesenchyme (**Figure 2 – figure supplement 3d**), the 610 genes significantly upregulated in Adult vs Aged CD90$^+$ cells were also enriched for 'Mitochondrial ATP synthesis coupled proton transport' and 'Oxidative phosphorylation'. Using the UCell method(Andreatta and Carmona, 2021), we found significantly reduced Complex I gene scoring with age in the suture mesenchyme and the periosteal CD90$^+$ cells (**Figure 2 – figure supplement 3e-f**). These results, coupled with live measurements of mitochondrial membrane potential (**Fig. 1i-j**), indicate an age-related decline in mitochondrial metabolism and electron transport chain (ETC) complexes within CD90$^+$ cells of the whole periosteum and suture mesenchyme. Importantly, we did not observe distinct changes in the signature of Glycolysis or lipid metabolism across age groups in CD90$^+$ cells in the osteogenic compartments (**Figure 2 – figure supplement 3j-m**).

Analysis with the CellChat package(Jin et al., 2021) shows inferred signaling heterogeneity within the periosteal CD90$^+$ population (**Figure 2 – figure supplement 4a**). Cells within the smaller CD90$^+$ cluster (cluster 3, **Figure 2 – figure supplement 1a-d**) receive incoming Wnt-signals and also Periostin-signalling, which upregulates Wnt/β-catenin pathway activity in osteogenesis(Lv et al., 2015; Zhang et al., 2017). This cluster is significantly upregulated for markers of osteogenic differentiation such as Runx2, Mme (Osteonectin/CD10)(Ding et al., 2020; Granéli et al., 2014), Bgn(Ye et al., 2012), Mgp(Zhang et al., 2019), Gas6(Shiozawa et al., 2010), and Igbp7(Zhang et al., 2018), and is hereby termed the P1 CD90$^+$ population. Cells within the larger cluster (clusters 0 & 1) both send and receive Periostin and Wnt-signals, and is significantly upregulated for markers associated with stemness, such as Klf4 and

Aldh1a3(Wanandi et al., 2018), and negative regulators of osteogenic differentiation such as Bmp3(Kokabu et al., 2012), and Ccn3(Matsushita et al., 2013). Hereby these cells were termed P2 CD90^{+}.

The periosteal P2 CD90^{+} cell population shifts with age between two categories, named 'Igf1^{high}' and 'Igf1^{low}' in relation to their differential expression of Igf1 (**Fig. 2a-c**). Igf1 is linked to bone anabolism through its dual roles in enhancing Wnt-signaling and exerting metabolic effects(Locatelli and Bianchi, 2014). Young P2 CD90^{+}cells were restricted to the Igf1^{high} grouping (92.0%), while Aged CD90^{+} cells were predominantly in the Igf1^{low} grouping (66.3%) (**Fig. 2a-b**). Adult CD90^{+} cells were more evenly split (56.8% Igf1^{high}, 43.2% Igf1^{low}). Igf1^{low} cells were significantly upregulated for 85 genes (**Fig. 2c**), linked 'Cell motility', driven by genes such as Mmp14(Almalki and Agrawal, 2016) and Itgb7(Novoseletskaya et al., 2020). Igf1^{high} cells were significantly upregulated for 204 genes linked to 'Collagen-containing extracellular matrix', 'Oxidative phosphorylation' and 'Respiratory chain complex'. The enrichment is driven by significantly upregulated genes such as NADH dehydrogenase genes (mt-Nd1 & mt-Nd2), Col1a1, Sparc.

Likewise, in the suture mesenchyme, analysis of inferred cellular state and function (**Fig. 2d-e**) revealed a distinct subpopulation of CD90^{+} cells. This group of cells was significantly upregulated for 855 genes linked to 'Mitotic cell cycle' and 'ATP-dependent activity'. In Young mice, this population, termed 'Proliferating' cells, represented 48.72% of the CD90^{+} population, whereas it markedly declined to around 8% in both Adult and Aged mice (**Fig. 2d-e**).

When comparing CD90^{+} cell populations between every two age stages, several genes are conserved in all three comparisons (**Figure 2 – figure supplement 4b, c**), including: Cavin1(Volonte and Galbiati, 2020), S100a4(Wagner et al., 2009), Vcan(S. Li et al., 2019), Mmp14(Haage et al., 2014), and Nsg1(Wiese et al., 2019). Each of these genes has been identified as being involved in senescence or the cellular response to aging tissues. Likewise, in the suture CD90^{+} cells, conserved aging-related genes included senescence-related genes Pim1(Yang et al., 2017), Pdcd4(Kang et al., 2002); and the senescence-associated secretory profile (SASP) gene Ccl6(Gillispie et al., 2021). Further comprehensive senescence signature confirms the significant increase associated with age in CD90^{+} cells (**Figure 2 – figure supplement 4d**). Also conserved were: pro-bone resorption Mmp9(Zhu et al., 2020), and adipogenesis-related genes ApoD(Provost et al., 1991) and Slc16a3 (MCT4)(Petersen et al., 2017).

Aging calvarial CD90^{+} cells undergo a decline in energy supply, proliferation, Wnt/β-catenin, and ECM remodeling capacity

Next, we aimed to investigate the transcriptionally-identified decline in energy supply, proliferation, Wnt/β-catenin pathway, and migration in CD90^{+} cells at the protein level through immunofluorescent staining of marker proteins.

The catalytic beta subunit of ATP synthase (ATPB), an essential component of the mitochondrial ATP synthesis machinery, serves as a marker for assessing energy-related mitochondrial function(H.-S. Li et al., 2019). Cellular ATPB levels in suture mesenchyme CD90^{+} cells is similar across age groups, but in the periosteal CD90^{+} cells, ATPB level is lower in Aged mice compared to Adults (**Fig. 2f, l**). Phosphorylated AMPK (phospho-AMPK), a sensor of ATP depletion(Gowans and Hardie, 2014), exhibits a negative correlation with energy availability. In both periosteum and suture CD90^{+} cells, mean phospho-AMPK was significantly higher in Aged mice (**Fig. 2g, l**). Taken together, these findings suggest a potential deficiency in oxidative phosphorylation machinery in CD90^{+} cells with age.

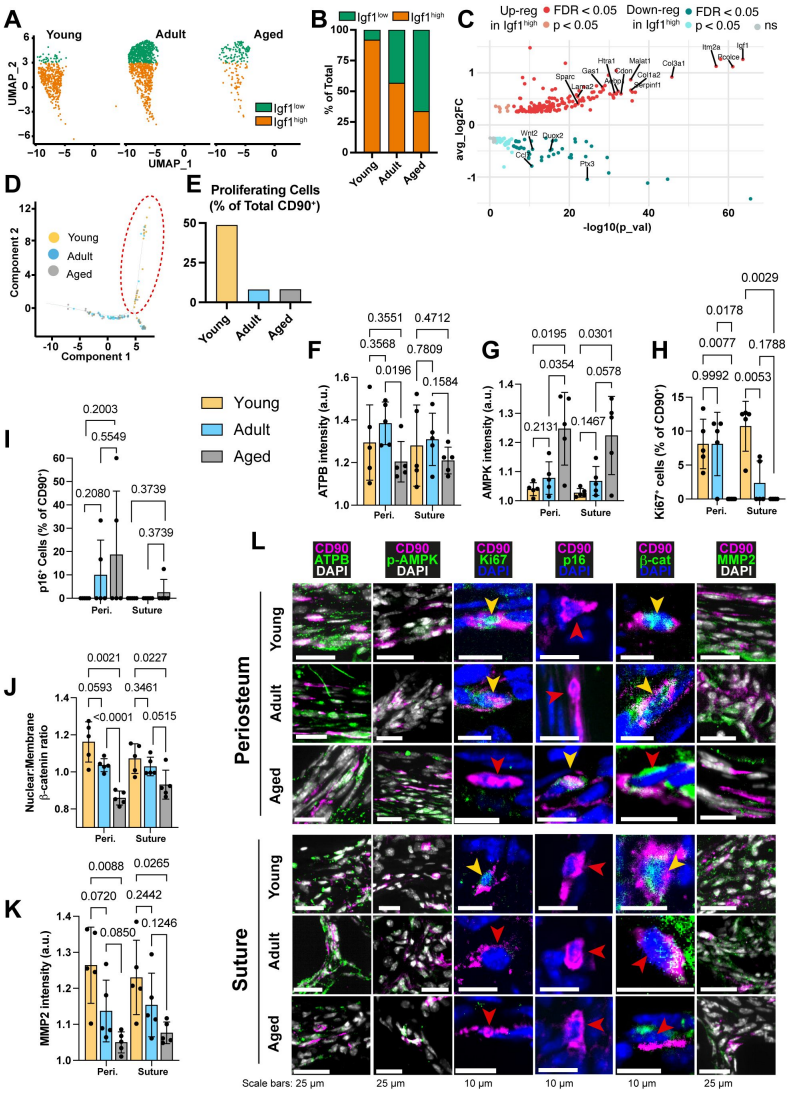

Figure 2.

Transcriptomic and protein expression characterization of CD90$^+$ cells during aging.

(**A**) UMAP-plot showing the distribution of a subpopulation of CD90$^+$ periosteal cells with aging, categorized as Igf1^{high} (orange) and Igf1^{low} (green). (**B**) Percentage of the total CD90$^+$ subpopulation categorized as either Igf1^{high} or Igf1^{low}. Young: Igf1^{high} (n = 425 cells), Igf1^{low} (n = 37); Adult: Igf1^{high} (n = 553), Igf1^{low} (n = 421); Aged: Igf1^{high} (n = 61), Igf1^{low} (n = 120). (**C**) Volcano plot showing differentially-expressed genes in the comparison of all Igf1^{high} cells (n = 1039) against all Igf1^{low} cells (n = 578). Statistical analysis: Wilcoxon Rank Sum test via the FindMarkers function in the Seurat R package; upregulated (pink: $p < 0.05$; red: False discovery rate (FDR)-adjusted $p < 0.05$), downregulated (cyan: $p < 0.05$; teal: FDR-adjusted $p < 0.05$), and non-significant (ns) genes are displayed on axes of log fold expression (Igf1^{high} over Igf1^{low}) (y, avg_log2FC) against p transformed as its negative log value. (**D**) Dimensional reduction plot (Monocle package(Trapnell et al., 2014)) of suture mesenchyme CD90$^+$ populations in Young (yellow), Adult (blue), and Aged (grey) mice. (**E**) Percentage of CD90$^+$ categorized as 'proliferating' (inside red dashed ellipse) vs 'non-proliferating' (outside ellipse). Young: proliferating (n = 19 cells), nonproliferating (n = 20); Adult: proliferating (n = 5), non-proliferating (n = 57); Aged: proliferating (n = 5), nonproliferating (n = 56). (**F**) Normalized intensity staining for ATPB in CD90$^+$ cells. (**G**) Normalized intensity staining for phospho-AMPK in CD90$^+$ cells. (**H**) Proportion of CD90$^+$ cells that are Ki67$^+$. (**I**) Proportion of CD90$^+$ cells that are p16$^+$. (**J**) Ratio of Nuclear:Membrane β-catenin staining in CD90$^+$ cells. (**K**) Normalized intensity staining for MMP2 in CD90$^+$ cells. For all plots (**F-K**), Young (yellow), Adult (blue), Aged (grey), periosteum (3 left columns), suture mesenchyme (3 right columns), statistical testing: Welch's tests in n = 5 mice per condition. (**L**) Representative images depicting CD90 (magenta), DAPI (grey/blue as indicated by column), and functional markers (green) as indicated by column. Yellow arrows indicate marker-high/positive cells; red arrows indicate marker-low/negative cells.

Ki67 marks active proliferation, while cyclin-dependent kinase inhibitors p16 is involved in various cellular functions and is linked to senescence and aging in several tissues(Safwan-Zaiter et al., 2022; Stein et al., 1999, p. 199). Calvarial $CD90^+$ cells were assessed for nuclear expression of the two markers. $Ki67^+$ $CD90^+$ cells were observed in the periosteum of both Young and Adult mice but not in Aged mice. In the suture, the proportion of $Ki67^+$ $CD90^+$ was significantly higher in Young than in Adult mice with none detected in Aged mice (**Fig. 2h, l**). The proportion of $p16^+$ $CD90^+$ cells appeared to increase with Age (**Fig. 2i, l**). Together, nuclear p16 expression and a lack of Ki67 expression suggests that Aged $CD90^+$ cells are not actively proliferating, corroborating inferred findings from transcriptomics.

Next, we assessed the level of nuclear β-catenin, an indicator of Wnt/β-catenin pathway activation. Aged $CD90^+$ cells in both periosteum and suture mesenchyme showed significantly reduced nuclear localization of β-catenin compared to Young (**Fig. 2j, l**). Only Adult $CD90^+$ cells in the periosteum had significantly higher nuclear β-catenin when compared to Aged mice.

Finally, in both periosteum and suture mesenchyme, Aged $CD90^+$ cells expressed the lowest amounts of MMP2 (**Fig. 2k-l**). While MMP2 is a secreted protein, only intracellular protein was quantified, to enable the analysis to be restricted to MMP2 expressed by $CD90^+$ cells alone. It corroborates the downregulation of genes associated with migration in the transcriptomic analysis.

Overall, our protein-level analysis largely aligns with the transcript-level findings, insinuating that age-related alterations in periosteum and suture mesenchyme, and consequently bone repair, could be considerably impacted by metabolic differences within the CD90-expressing population.

Given that increasing the number of $CD90^+$ cells inside the defect alone produced only a minor improvement to Aged bone repair, we developed a systemic approach to target cellular metabolism. We aimed to combine this systemic approach with the use of the Wnt3a-bandage to ultimately facilitate the supply of competent osteogenic cells for bone repair.

Intermittent Fasting improves bone health and bone repair in Aged mice

Intermittent fasting (IF) can improve metabolic control by various measures(Longo et al., 2021) and bone mineral density in adult animals (Veronese and Reginster, 2019). We produced critical-sized calvarial defects in Aged mice, treated them with Wnt3a-bandages and inactive controls. These mice were then maintained on an alternating-day diet of self-limited feeding and complete fasting, hereby termed the intermittent fasting diet. Importantly, over the course of a week, mice intake the same amount of food during the IF diet as during the self-limited feeding controls (*ad libitum*, AL) diet (**Figure 3 – figure supplement 1a-c**), and is therefore distinct from an overall reduction in calories (i.e., caloric restriction). Fasting blood glucose levels and the area under the curve (AUC) from the intraperitoneal glucose tolerance test (IPGTT) were significantly lower in mice following an IF diet compared to those on an AL diet (**Figure 3 – figure supplement 1d-g**). These results indicate improved insulin sensitivity, enhanced glucose metabolism, and better overall metabolic control(Andrikopoulos et al., 2008; McGuinness et al., 2009; Nakrani et al., 2024).

Remarkably, Aged mice subjected to the IF diet repaired 59.24% of the defect within 10 weeks under the inactive bandage, higher even than that of Adult mice + inactive bandage (**Fig. 1o-p**). With the addition of the Wnt3a-bandage, bone repair area was increased to 67.10%. This combination approach of the local Wnt3a-bandage and post-fracture IF results in Aged calvarial bone repair rivaling that of Young mice. The IF diet also significantly increased the solid bone proportion (BV/TV) (**Fig. 3a-b**), making it similar to the much younger Adult bone.

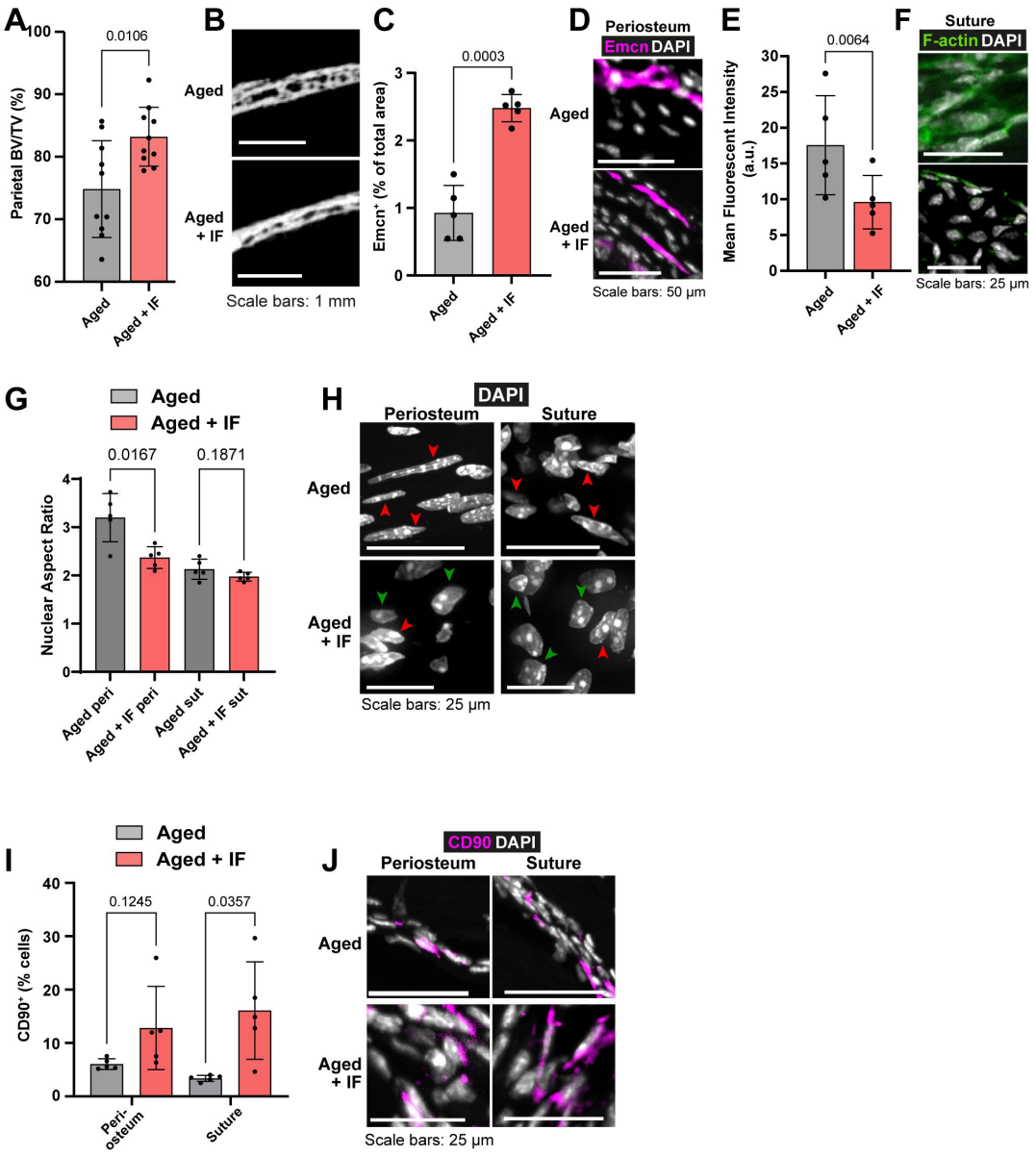

Figure 3.

The impact of Intermittent Fasting on Aged bone structure and osteogenic tissues.

(**A**) Parietal Bone Volume/Total Volume (BV/TV) and (**B**) representative transverse micro computed tomography (microCT) of Aged mice undergoing intermittent fasting. Statistical analysis: Welch's test in n = 10 mice per condition. (**C**) Mean cross sectional area taken up by Endomucin (Emcn)-positive vascular cells and (**D**) representative immunofluorescence staining of Emcn (magenta) and DAPI (grey) in the periosteum of Aged mice undergoing intermittent fasting. Statistical analysis: Welch's tests in n = 5 mice per condition. (**E**) Mean Phalloidin-488 fluorescence intensity and (**F**) representative images for filamentous actin (F-actin, green) and DAPI (grey) in the suture mesenchyme of Aged mice undergoing intermittent fasting. Statistical analysis: Welch's test in n = 5 mice per condition. (**G**) Nuclear aspect ratio (long axis divided by short axis) of cells within the periosteum and suture mesenchyme and representative DAPI-stained nuclei (**H**) of Aged mice undergoing intermittent fasting. Statistical analysis: Welch's tests in n = 5 mice per condition. Green and red arrows highlight rounded and elongated nuclei, respectively. (**I**) Percentage of $CD90^+$ cells within the periosteum (left columns) and suture (right columns), expressed relative to the total cellular number ($DAPI^+$) in Aged mice undergoing intermittent fasting. Representative images (**J**) show CD90 immunostaining (magenta) and DAPI (grey). Statistical analysis: Welch's tests in n = 5 mice. In panels **A**, **C**, **E**, **G and I**, columns in grey are data previously shown in **Fig. 1**, reproduced here for reference. In panels **B**, **D**, **F**, **H**, **J**, Aged control representative images previously shown in **Fig. 1** are reproduced here for reference.

Intermittent Fasting reverses aspects of cellular and tissue aging in the periosteum and suture mesenchyme

Compared to Aged mice, in Aged + IF, Emcn$^+$ vascularity in the periosteum was significantly increased (**Fig. 3c-d**). Additionally, in the suture mesenchyme, actin cytoskeleton staining was markedly decreased (**Fig. 3e-f**), along with a significant reduction in age-related nuclear elongation both in the suture and periosteum (**Fig. 3g-h**). The IF diet did not impact Lamin B1 protein levels (**Figure 1 – figure supplement 2i-j**). The IF diet appeared to elevate the proportion of CD90$^+$ cells in the periosteum, while in the suture it significantly increased compared to AL (**Fig. 3i-j**).

To gain a deeper understanding of how IF affects Aged CD90$^+$ cells, we conducted further scRNAseq analysis. We maintained Aged mice on the alternating-day IF diet for 10 weeks before performing scRNAseq on the periosteum and suture mesenchyme (Source Data 2 – Supplementary Tables 9,10,12).

Comparing CD90$^+$ cells of Aged periosteal post IF diet with Aged AL diet controls revealed 98 significantly upregulated genes (**Fig. 4a**). These were enriched mainly for 'Response to cytokine' and similar GO terms. Of the genes upregulated with the IF diet, 10 were individually statistically significant when adjusting for false discovery rate ($q < 0.05$). Ccl11 and Duox2 are involved in osteogenic stem cell migration(Smith et al., 2012; Tyurin-Kuzmin et al., 2016), Tnfaip6 and Ccl7 in immunomodulation(Li et al., n.d.; Rafei et al., 2008), Ptx3 is involved in both processes(Cappuzzello et al., 2016), and Actg1 in cytoskeletal structure(Dugina et al., 2009; Simiczyjew et al., 2014). Zbtb16, encoding transcription factor PLZF, regulates osteogenesis by acting upstream of master osteoblast transcription factor Runx2 (**Figure 2 – figure supplement 3i**)(Agrawal Singh et al., 2019; Liu and Lee, 2013).

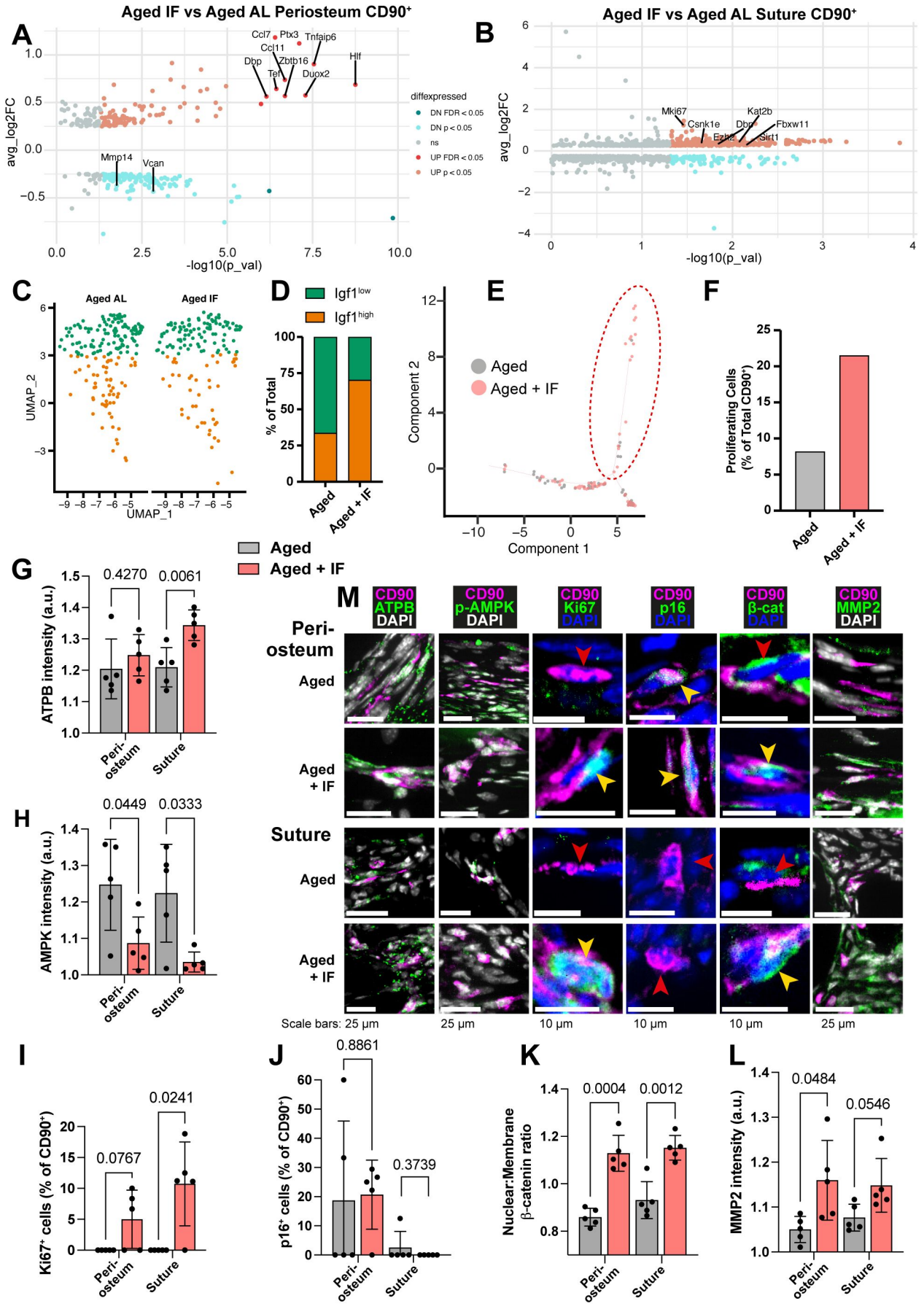
A
Aged IF vs Aged AL Periosteum CD90+
avg_log2FC
-log10(p_val)
Ccl7
Ptx3
Tnfaip6
Ccl11
Dbp
Zbtb16
Hlf
Tef
Duox2
Mmp14
Vcan
diffexpressed
DN FDR < 0.05
DN p < 0.05
ns
UP FDR < 0.05
UP p < 0.05
B
Aged IF vs Aged AL Suture CD90+
Mki67
Csnk1e
Kat2b
Dbp
Fbxw11
Ezh2
Sirt1
C
Aged AL
Aged IF
UMAP_2
UMAP_1
D
Igf1low
Igf1high
% of Total
Aged
Aged + IF
E
Aged
Aged + IF
Component 2
Component 1
F
Proliferating Cells (% of Total CD90+)
Aged
Aged + IF
G
ATPB intensity (a.u.)
0.4270
0.0061
Peri-osteum
Suture
H
AMPK intensity (a.u.)
0.0449
0.0333
Peri-osteum
Suture
M
CD90 ATPB DAPI
CD90 p-AMPK DAPI
CD90 Ki67 DAPI
CD90 p16 DAPI
CD90 β-cat DAPI
CD90 MMP2 DAPI
Peri-osteum
Aged
Aged + IF
Suture
Aged
Aged + IF
Scale bars: 25 μm
25 μm
10 μm
10 μm
10 μm
25 μm
I
Ki67+ cells (% of CD90+)
0.0767
0.0241
Peri-osteum
Suture
J
p16+ cells (% of CD90+)
0.8861
0.3739
Peri-osteum
Suture
K
Nuclear:Membrane β-catenin ratio
0.0004
0.0012
Peri-osteum
Suture
L
MMP2 intensity (a.u.)
0.0484
0.0546
Peri-osteum
Suture

Figure 4.

Transcriptomic and protein expression characterization of Aged CD90$^+$ cells during intermittent fasting.

(**A**) Volcano plot showing differentially-expressed genes in the comparison of the Aged CD90$^+$ population in the periosteum and (**B**) suture during intermittent fasting (IF) vs the *ad libitum* (AL) diet. Statistical analysis: Wilcoxon Rank Sum test via the FindMarkers function in the Seurat R package. Upregulated (pink: p < 0.05; red: FDR-adjusted p < 0.05), downregulated (cyan: p < 0.05; teal: FDR-adjusted p < 0.05), and nonsignificant (ns) genes are displayed on axes of log fold expression (IF over AL) (y, avg_log2FC) against p transformed as its negative log value. Periosteum: Aged IF (n = 194 cells); Aged AL (n = 242 cells). Suture: Aged IF (n = 79 cells); Aged AL (n = 61 cells). (**C**) UMAP-plot showing the distribution of a subpopulation of CD90$^+$ periosteal cells during intermittent fasting in Adult and Aged mice. AL cells are data previously shown, reproduced here for reference. (**D**) Percentage of the total cells categorized as either Igf1^{high} or Igf1^{low}. Aged + IF: Igf1^{high} (n = 104), Igf1^{low} (n = 44). (**E**) Plot (Monocle package) of suture CD90$^+$ populations. (**F**) Percentage of CD90$^+$ categorized as 'proliferating' (inside red dashed ellipse) vs 'non-proliferating' (outside ellipse) in Aged mice undergoing intermittent fasting. AL cells and chart are data previously shown, reproduced here for reference. Statistical analysis: two-tailed binomial test based on expected observed proportions between groups. Adult + IF: proliferating (n = 30 cells), non-proliferating (n = 86); Aged + IF: proliferating (n = 17), non-proliferating (n = 62). (**G**) Normalized intensity staining for ATPB in CD90$^+$ cells. (**H**) Normalized intensity staining for phospho-AMPK in CD90$^+$ cells. (**I**) Proportion of CD90$^+$ cells that are Ki67$^+$. (**J**) Proportion of CD90$^+$ cells that are p16$^+$. (**K**) Ratio of Nuclear:Membrane β-catenin staining in CD90$^+$ cells. (**L**) Normalized intensity staining for MMP2 in CD90$^+$ cells. For all plots (**G**-**L**), Aged + IF (red), Aged control (grey), periosteum (2 left columns), suture mesenchyme (2 right columns), statistical testing: Welch's tests in n = 5 mice per condition. (**M**) Representative images depicting CD90 (magenta), DAPI (grey/blue as indicated by column), and functional markers (green) as indicated by column). Yellow arrows indicate marker-high/positive cells; red arrows indicate marker-low/negative cells. In panels **C**-**M,** data and representative images of Aged controls from previous figures are reproduced here for reference.

In the suture mesenchyme, analysis of 297 significantly upregulated genes in Aged CD90$^+$ cells during the IF diet compared to Aged AL control (**Fig. 4b**) showed enrichment in 'Mitotic cell cycle' and 'Protein phosphorylation'. Notably, the proliferation marker Mki67 is significantly upregulated, with a threefold increase in Aged periosteum CD90$^+$ cells during intermittent fasting compared to ad libitum diet.

Intermittent fasting also showed an upregulation of Dbp, Tef, and Hlf, an effect that was conserved between osteoprogenitor compartments in Aged mice. (**Figure 2 – figure supplement 3g-h**, Supplementary File 2). These genes are part of the transcriptional effectors of the circadian transcriptional network (Gachon et al., 2004), which act by binding to D-box sites in gene promoters (Yoshitane et al., 2019). In suture Aged CD90+ cells during the IF diet, Dbp alone is significantly upregulated against Aged AL CD90+ cells. Several other genes under circadian control (e.g., Sirt1, Kat2b, Csnk1e, Ezh2, Fbxw11, Ucp2; all p < 0.05) are upregulated in Aged suture CD90+ cells during the IF diet. This corresponds to an enrichment of the transcriptional targets of Clock/Per2/Arntl, which together form the network of transcription factors responsible for maintaining circadian rhythms.

In **Fig. 2a**, a shift in the periosteal CD90$^+$ was noted with aging. Aged AL had relatively fewer osteogenic Igf1^{high} cells, and more of Igf1^{low} cells. Contrary, Aged + IF CD90$^+$ cells were split 70.3% Igf1^{high}, 29.7% Igf1^{low} (**Fig. 4c-d**), positioning them between Young and Adult distributions. 2 of the 14 genes identified as conserved markers of periosteal CD90$^+$ cell aging (**Figure 2 – figure supplement 4b**), Vcan, Mmp14, were significantly downregulated in Aged + IF. The proportion of 'proliferating' CD90$^+$ suture cells increased in Aged + IF (**Fig. 4e-f**). Further, 1 of the 32 conserved genes of the aging suture (**Figure 2 – figure supplement 4c**), actin-associated

Tpm1(Li et al., 2022), was significantly downregulated in Aged + IF. Wnt2, found to be downregulated with aging, is significantly upregulated in the suture CD90$^+$ population of Aged + IF (Source Data 2 – Supplementary Table 10).

To validate diet-induced changes in CD90$^+$ cells, we conducted immunostaining for protein markers in histological sections. ATPB, marker of ATP synthase, appeared elevated in periosteum and significantly increased in suture mesenchyme CD90$^+$ cells following IF (**Fig. 4g-m**). This suggests an increase in cellular ATP levels, which is supported by decreases in ATP-deficiency sensor phospho-AMPK in periosteal and suture CD90$^+$ cells (**Fig. 4h, m**). While proliferating cells were absent in Aged mice, we observed Ki67$^+$ cells in samples of the periosteum of 3 out of 5 Aged + IF mice and in 4 out of 5 Aged + IF mice for suture mesenchyme (**Fig. 4i, m**). Conversely, p16 was similar in periosteum and suture mesenchyme (**Fig. 4j, m**). Wnt/βcatenin pathway activity, as measured by the relative nuclear localization of β-catenin, was significantly increased within the CD90$^+$ cells of both the periosteum and suture (**Fig. 4k, m**). Lastly, expression of the motility marker MMP2 in CD90$^+$ cells was markedly increased over Aged controls in the periosteum, and just under statistical significance in the suture mesenchyme (**Fig. 4l, m**).

Together, these findings suggest that IF rejuvenates aged CD90$^+$ cells, in part, by enhancing proliferation, immune response, ECM remodeling, Wnt/β-catenin pathway, and metabolism, including increased ATP levels and decreased AMPK levels.

Short term metabolic approaches rejuvenate the calvarial osteogenic compartments of Aged mice and promote bone repair

IF diet likely enhances energy supply to CD90$^+$ cells and their function through various mechanisms. We next aimed to determine if specific underlying mechanisms alone could replicate the effects of IF on aged bone repair. Our analysis revealed that both aging and IF affect the NAD$^+$ biosynthesis pathway and gut microbiota. Thus, we targeted these aspects to investigate their potential to enhance aged bone repair.

NMN supplementation improves bone repair in Aged mice

NAD$^+$ is a multifunctional enzyme co-factor with roles including protein deacetylation, histone modification, intracellular signaling, and redox reactions (Covarrubias et al., 2021). It is also crucial in oxidative phosphorylation(Stein and Imai, 2012), cycling between oxidized and reduced forms (NAD$^+$/NADH) in the mitochondria as part of the electron transport chain. Our analysis of inferred NAD$^+$-dependent pathway activity (**Figure 2 – figure supplement 3d**, Source Data 2 – Supplementary Table 4), live mitochondrial activity (**Fig. 1i-j**), and markers of cellular energy levels (**Fig. 2f, g, l**) demonstrated distinct changes to metabolism with age. Yet, little is known about the effect of NAD$^+$-coupled cellular metabolism on the composition of aging calvarial periosteum and suture mesenchyme and its influence over Aged bone repair.

To increase cellular levels of NAD$^+$ systemically, we orally dosed Aged mice with nicotinamide mononucleotide (NMN), an immediate precursor to NAD$^+$ in the 'salvage' pathway(Yang and Sauve, 2016). During a pilot study involving oral NMN supplementation (600 mg/kg, 3 times within 1 week) in Adult mice, the mitochondrial membrane potential, as measured by TMRM staining, within the cells of the periosteum was significantly increased (**Fig. 5a, b**). NMN was then administered to Aged mice for the 2 weeks prior and 2 weeks following calvarial defect surgery.

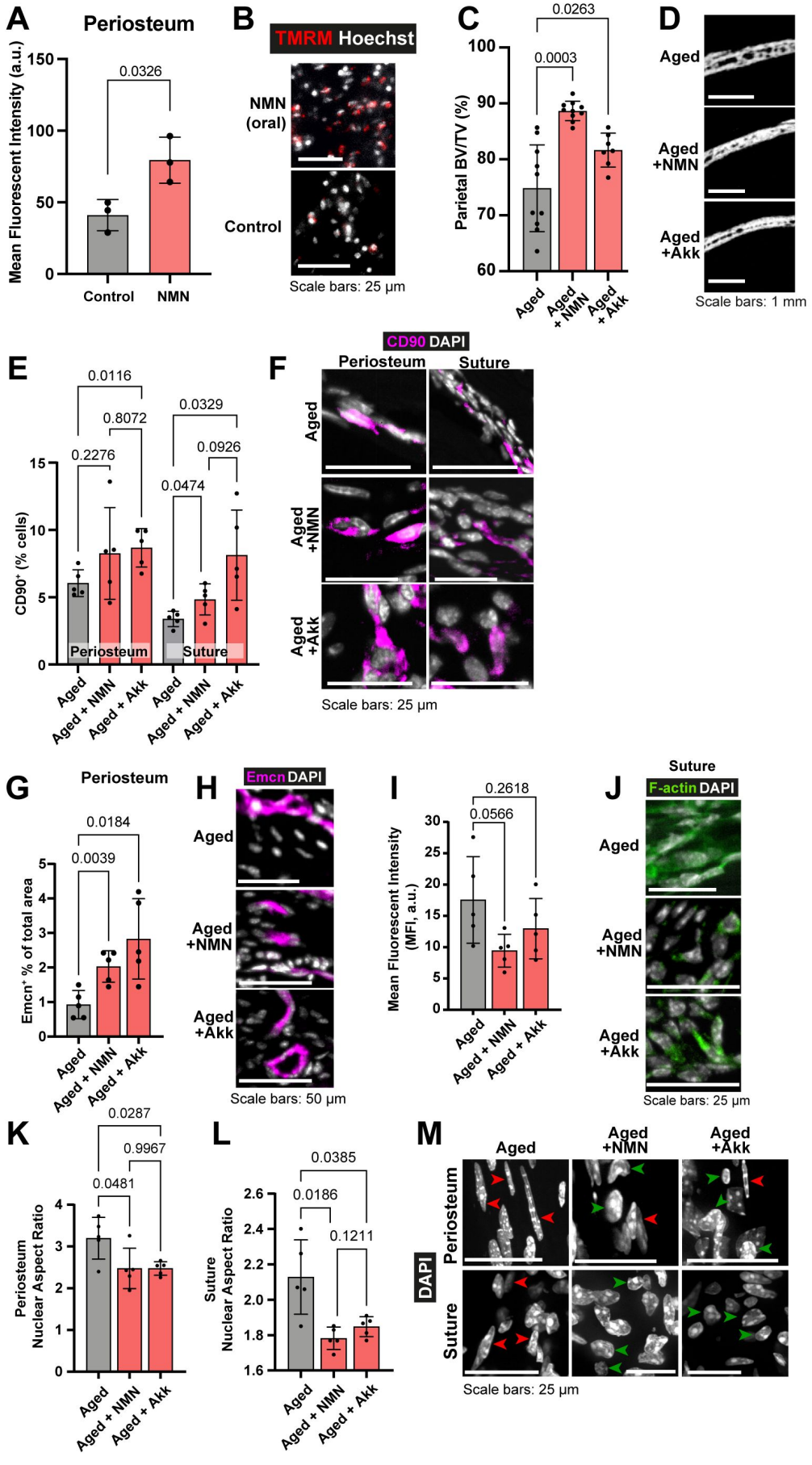
A
Periosteum
Mean Fluorescent Intensity (a.u.)
0.0326
Control
NMN
B
TMRM Hoechst
NMN (oral)
Control
Scale bars: 25 µm
C
0.0263
0.0003
Parietal BV/TV (%)
Aged
Aged + NMN
Aged + Akk
D
Aged
Aged +NMN
Aged +Akk
Scale bars: 1 mm
E
0.0116
0.8072
0.2276
0.0329
0.0926
0.0474
CD90+ (% cells)
Periosteum
Suture
Aged
Aged + NMN
Aged + Akk
F
CD90 DAPI
Periosteum
Suture
Aged
Aged +NMN
Aged +Akk
Scale bars: 25 µm
G
Periosteum
0.0184
0.0039
Emcn+ % of total area
Aged
Aged + NMN
Aged + Akk
H
Emcn DAPI
Aged
Aged +NMN
Aged +Akk
Scale bars: 50 µm
I
0.2618
0.0566
Mean Fluorescent Intensity (MFI, a.u.)
Aged
Aged + NMN
Aged + Akk
J
Suture
F-actin DAPI
Aged
Aged +NMN
Aged +Akk
Scale bars: 25 µm
K
0.0287
0.9967
0.0481
Periosteum Nuclear Aspect Ratio
Aged
Aged + NMN
Aged + Akk
L
0.0385
0.0186
0.1211
Suture Nuclear Aspect Ratio
Aged
Aged + NMN
Aged + Akk
M
Aged
Aged +NMN
Aged +Akk
Periosteum
Suture
DAPI
Scale bars: 25 µm
N
0.2392
Akkermansia Abundance (%)
Aged
Aged + IF

Figure 5.

The impact of Nicotinamide mononucleotide (NMN) and *Akkermansia muciniphila* (Akk) supplementation on Aged bone structure and osteogenic tissues.

(**A**) Mean intensity and (**B**) representative images of live TMRM staining (red) and Hoechst (grey) in Adult mice controls and Adult mice dosed with 600 mg/kg NMN by oral gavage, samples of periosteum *ex vivo*. Statistical analysis: Welch's test in tissue from n = 3 mice per condition. (**C**) Parietal Bone Volume/Total Volume (BV/TV) and (**D**) representative transverse micro-computed tomography (microCT) sections in Aged mice supplemented with NMN or *Akkermansia*. Statistical analysis: Welch's tests in n = 10 (Aged, Aged + NMN) and n = 7 (Aged + Akk) mice. (**E**) Percentage of $CD90^+$ cells within the periosteum (left columns) and suture (right columns), expressed relative to the total cellular number ($DAPI^+$) in Aged mice supplemented with NMN or *Akkermansia*. Representative images (**F**) show CD90 immunostaining (magenta) and DAPI (grey). Statistical analysis: Welch's tests in n = 5 mice per condition. (**G**) Mean cross sectional area taken up by Endomucin (Emcn)-positive vascular cells and (**H**) representative immunofluorescence staining of Emcn (magenta) and DAPI (grey) in the periosteum of Aged mice supplemented with NMN or *Akkermansia*. Statistical analysis: Welch's tests in n = 5 mice per condition. (**I**) Mean Phalloidin-488 fluorescence intensity and (**J**) representative images for filamentous actin (F-actin, green) and DAPI (grey) in the suture mesenchyme of Aged mice supplemented with NMN or *Akkermansia*. Statistical analysis: Welch's tests in n = 5 mice per condition. (**K-M**) nuclear aspect ratio (long axis divided by short axis) of cells within the (**K**) periosteum and (**L**) suture in Aged mice supplemented with NMN or *Akkermansia*, and (**M**) Representative DAPI-stained nuclei. Statistical analysis: Welch's tests in n = 5 mice per condition. Green and red arrows highlight rounded and elongated nuclei, respectively. (**N**) The relative abundance of bacteria identified as genus *Akkermansia* in 16S metagenomic profiling of Aged mice undergoing intermittent fasting vs. Aged controls. Statistical analysis: Welch's test in (Aged, n = 3; Aged + IF, n = 4) mice per condition. In panels **C-M**, data and representative images of Aged controls from previous figures are reproduced here for reference.

Parietal bone BV/TV was significantly increased in Aged animals that received NMN (Aged + NMN) (**Fig. 5c-d**). Remarkably, Aged bone repair was significantly increased to 54.32% with NMN with inactive bandages (**Fig. 1o-p**), marking a three-fold improvement over control Aged mice. Adding Wnt3a-bandage increased Aged + NMN repair to 71.18%, resulting in over 2.71 times greater area of new bone compared to Aged controls + Wnt3a-bandage, akin to repair in Adult mice + Wnt3a-bandage.

Next, we probed how NMN affects $CD90^+$ cells in the osteogenic compartments. In the suture mesenchyme, NMN treatment significantly increased $CD90^+$ cells compared to Aged control (**Fig. 5e-f**). In the periosteum, $CD90^+$ populations were unchanged. Additionally, compared to Aged controls, NMN supplementation significantly increased the vascularity of the periosteum (**Fig. 5g-h**), and approximately halved actin cytoskeletal staining intensity in the suture mesenchyme (**Fig. 5i-j**). Nuclei in the suture and periosteum were rounder and less elongated than Aged controls (**Fig. 5k-m**), making them morphologically resemble Adult nuclei.

Taken together, NMN treatment increased periosteal vascularity, impacted aspects of tissue mechanics of the periosteum and suture mesenchyme, leading to a significant improvement in bone repair in Aged mice.

Cyclical nutrient deprivation and NMN enhance mitochondrial function in osteogenic cells in vitro

Next, we aimed to model aspects of the effects of intermittent fasting and NMN supplementation on osteogenic cells, employing a cyclical nutrient deprivation regimen or NMN supplementation *in vitro* to elucidate aspects of their mechanisms of action. While this approach allows us to study

specific cellular responses, it is crucial to recognize that it cannot fully replicate the complex systemic effects of *in vivo* intermittent fasting.

We investigated the impact of these interventions on mitochondrial function in the MC3T3 calvarial osteoblastic cell line, a model commonly utilized to study osteogenesis *in vitro* and known for its ability to contribute to bone formation *in vivo*(Horowitz et al., 1994; Paine et al., 2018; Wang et al., 1999).

Alternating serum deprivation every hour (cyclical nutrient deprivation) in pyruvate and glucose-free medium induced the expression of genes related to mitochondrial biogenesis, fusion, mitophagy, and the unfolded protein response (**Fig. 6a**). This was accompanied by increased mitochondrial filament length and membrane potential, indicating enhanced mitochondrial function(McCarron et al., 2013; Westermann, 2012) (**Fig. 6b-e**). These findings are consistent with our *in vivo* observations of improved mitochondrial function in animals subjected to intermittent fasting. While cyclical nutrient deprivation enhanced mitochondrial membrane potential, it also reduced overall cellular ATP levels. However, inhibiting mitochondrial ATP production with oligomycin (a Complex V inhibitor)(Devenish et al., 2000) further diminished ATP levels, suggesting that mitochondrial respiration is a primary source of ATP in these cells (**Fig. 6f**).

Recognizing the role of mitochondrial dysfunction in aging, we investigated the impact of NMN on MC3T3 cells challenged with oligomycin and cultured in glucose and pyruvate-free medium. NMN treatment mitigated oligomycin-induced ATP loss and ROS production, enhanced cell proliferation, and prevented the loss of mitochondrial membrane potential (**Fig. 7a-c**). Holotomography microscopy revealed that NMN preserved the “rod-like/filament” morphology of mitochondria in oligomycin-treated cells (**Fig. 7d-e**). Furthermore, NMN restored the expression of genes related to mitochondrial biogenesis and dynamics, the unfolded protein response, and mitophagy, which were adversely affected by oligomycin (**Fig. 7f**). Ultimately, NMN enhanced the growth and differentiation potential of oligomycin-treated cells, as evidenced by increased ALP activity and mineralization (**Fig. 7g**).

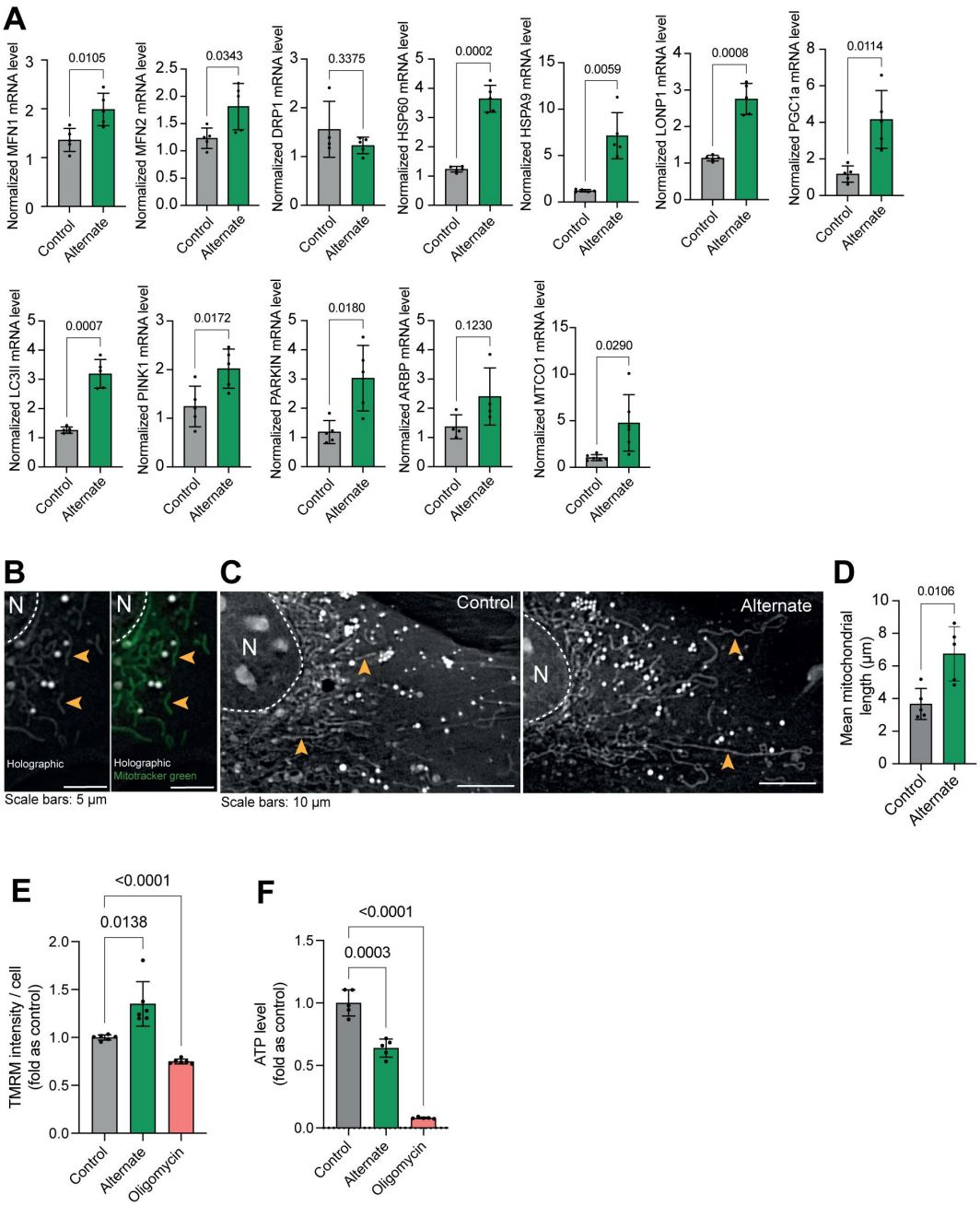

Figure 6.

Effect of hour-based alternate serum deprivation on mitochondria in MC3T3 cells in glucose-free media.

(**A**) Effect of alternate serum deprivation on gene expression involved in mitochondrial dynamics (MFN1, MFN2, DRP1), unfolded protein response (mtUPR) (HSP60, HSPA9, LonP1), biogenesis (PGC1a), structure (ARBP), mitophagy (LC3II, PINK1, PARKIN), and mitochondrial respiratory complex IV (MTCO1). Statistical analysis: Welch's test, n≥4/group. (**B**) Representative holographic images of mitochondrial shape (orange arrowheads), confirmed by Mitotracker green staining. (**C-D**) Effect of alternate serum deprivation on mitochondrial length. Statistical analysis: Welch's t-test of n=5/group, total mitochondria=425. (**E**) Effect of alternate serum deprivation and oligomycin on mitochondrial membrane potential. Statistical analysis: Welch's t-test of n=6/group with at least 481 cells/group. (**F**) Effect of alternate serum deprivation and oligomycin on ATP level. Statistical analysis: Welch's t-test of n≥5/group.

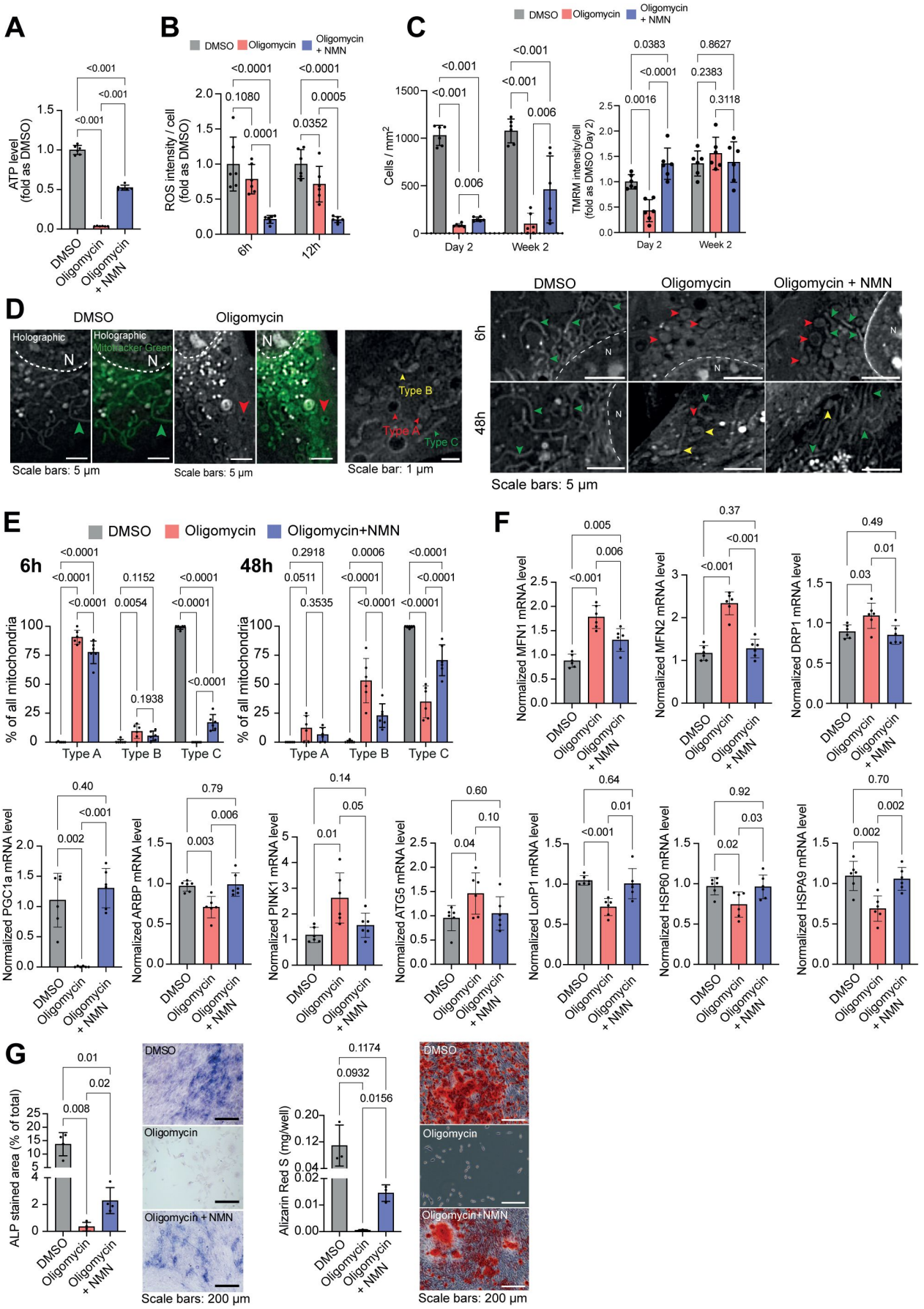
A
ATP level (fold as DMSO)
DMSO
Oligomycin
Oligomycin + NMN
B
ROS intensity / cell (fold as DMSO)
6h
12h
C
Cells / mm2
Day 2
Week 2
TMRM intensity/cell (fold as DMSO Day 2)
D
Holographic
Mitotracker Green
Type A
Type B
Type C
Scale bars: 5 µm
Scale bar: 1 µm
E
% of all mitochondria
6h
48h
F
Normalized MFN1 mRNA level
Normalized MFN2 mRNA level
Normalized DRP1 mRNA level
Normalized PGC1a mRNA level
Normalized ARBP mRNA level
Normalized PINK1 mRNA level
Normalized ATG5 mRNA level
Normalized LonP1 mRNA level
Normalized HSP60 mRNA level
Normalized HSPA9 mRNA level
G
ALP stained area (% of total)
Alizarin Red S (mg/well)
Scale bars: 200 µm

Figure 7.

Effect of NMN on oligomycin-induced mitochondrial damage in glucose-free MC3T3 cells.

(**A**) Effect of oligomycin ± NMN on ATP level in MC3T3 cells. Welch's test, n=6/group. (**B**) Effect of oligomycin ± NMN on ROS level MC3T3 cells. Welch's test, n=6/group. (**C**) Effect of oligomycin ± NMN on cell density (left) and mitochondrial membrane potential (right) over time in MC3T3. Welch's test, n=6/group. (**D**) Representative images of mitochondrial shape alteration by oligomycin exposure ± NMN supplementation. Dashed white line = nuclear membrane, N = nucleus. Observation by holotomography (grey levels) and confirmed with Mitotracker Green (green). Red arrowheads = Type A ("round"), yellow arrowheads = Type B ("intermediate"), and green arrowheads = Type C ("rod-like/filamentous") mitochondria. (**E**) Quantification of the effect of oligomycin ± NMN on mitochondrial shape. Each data point is a cell with at least 30 mitochondria. Welch's test n = 6 cells / group, total mitochondria=1821. (**F**) Effect of oligomycin ± NMN on gene expression involved in mitochondrial dynamics (MFN1, MFN2, DRP1), biogenesis (PGC1a), structure (ARBP), mitophagy (PINK1, ATG5), and unfolded protein response (mtUPR) (LonP1, HSP60, HSPA9). Welch's test, n≥5/group. (**G**) Effect of oligomycin ± NMN on osteoblastic differentiation. Staining of alkaline phosphatase (ALP) activity and mineralization (Alizarin Red S; ARS) of the matrix after two weeks of differentiation. Welch's test, n=4 (ALP) or 3 (ARS) / group.

In summary, our findings suggest that both cyclical nutrient deprivation and NMN supplementation can enhance mitochondrial function in osteogenic cells. NMN appears to protect against mitochondrial dysfunction induced by Complex V inhibition, highlighting its potential in mitigating the decline in osteogenic cell function.

Akkermansia muciniphila supplementation improves bone repair in Aged mice

The 'gut-bone axis' links food intake, gut microbiome and bone metabolism via circulating metabolites(Villa et al., 2017). Bacteria in the genus *Akkermansia* appeared to be elevated during Aged + IF compared to the AL control diet (**Fig. 5n**), consistent with previous studies that found similar results in young mice and human(Li et al., 2020; Özkul et al., 2019).

Akkermansia muciniphila is an anaerobic gut bacterium responsible for mucin turnover(Derrien et al., 2008), and a regulator of the intestinal mucus environment(Reunanen et al., 2015). The *Akkermansia* population exerts wider effects on the gut microbiome, and has been studied for its roles in metabolic health(Dao et al., 2016; Leite et al., 2021). We cultured *Akkermansia muciniphila* (Akk) under anaerobic conditions and calculated the viability of cryopreserved aliquots. Doses of live Akk (2 x 10^8 C.F.U.) were given by oral gavage three times per week in Aged mice for the 2 weeks prior and 6 weeks following calvarial defect surgery.

Our metagenomic analysis reveals that Firmicutes:Bacteroides ratio, indicator of microbiome composition(Magne et al., 2020), is significantly increased in Aged mice with *Akkermansia* supplementation (Aged + Akk) (**Figure 5 - figure supplement 1a**). Furthermore, the proportion of gut bacteria belonging to family *Lachnospiraceae,* indicative of the capacity of the gut bacteria to produce short chain fatty acids (SCFAs), is significantly elevated in Aged + Akk (**Figure 5 - figure supplement 1b**). SCAFs enter the host bloodstream and exert epigenetic effects primarily through histone modification, which might have implication on cell function and differentiation(Licciardi et al., 2011).

Similar to NMN treatment, parietal bone BV/TV was significantly increased Aged +Akk (**Fig. 5c-d**). Aged bone repair was significantly increased to 43.76% with Akk and inactive bandages (**Fig. 1o-p**). In combination with the Wnt3a bandage effect, bone repair in Aged + Akk further

increased to 64.84%. This level of bone repair is similar to that of Young mice with Wnt3a-bandage (Supplementary File 1). Aged + Akk also produced striated bone, similar in structure to Young mice, under both Wnt3a and control bandages (**Figure 1 - figure supplement 3d**).

Aged + Akk led to a significant increase in $CD90^+$ cells in the periosteum and suture mesenchyme (**Fig. 5e-f**) and a threefold increase in the $Emcn^+$ vascularity area of the periosteum (**Fig. 5g-h**). Compared to Aged controls, in Aged + Akk, actin cytoskeletal staining intensity appeared reduced (not significant) in the suture mesenchyme (**Fig. 5i-j**), and nuclei in both the suture and periosteum appeared rounder and less elongated (**Fig. 5k-m**). Accordingly, the nuclear morphology of Aged + Akk cells resembles that of younger mice.

In summary, we have revealed a multifaceted decline in $CD90^+$ cells with aging, which can be locally counteracted by a Wnt3a-bandage or systemically enhanced through an IF diet. Moreover, systemic interventions, acting via the gut and on shorter terms, can improve $CD90^+$ populations, osteogenic compartments, and significantly enhance bone repair in Aged mice. Combining the Wnt3a-bandage with systemic interventions targeting cellular energy supply, proliferation, migration, and Wnt signaling presents coordinated and clinically relevant approaches, promising rejuvenation of Aged bone repair comparable to younger individuals.

Discussion

Here we report on systemic interventions that can reverse aspects of the aging osteogenic tissue to promote effective calvarial bone repair (**Appendix 2 – figure 1**).

While extensive research focuses on long bones(Yates et al., 2007), there is a significant gap in understanding aging in flat bones. Our study on the calvaria demonstrates a clear decline in both bone structure and healing capacity with age. We observed a progressive decline in periosteal blood vessel density beginning in adulthood. Microvascular rarefaction is increasingly recognized as a driver, rather than merely a consequence, of hallmarks of cellular aging, such as cellular senescence(Goligorsky, 2010; Lähteenvuo and Rosenzweig, 2012). Indeed, previous studies inducing vasculature loss in young animals accelerates cellular aging(Chen et al., 2021), while mitigating this vascular decline preserves tissue health and delays aging(Grunewald et al., 2021). Vascularization and modulation of blood flow are critical for calvarial bone repair (Holstein et al., 2011; Huang et al., 2015; Ren et al., 2024). Early in the repair process, periosteal blood vessels grow independently of osteoprogenitor cells, establishing a supportive environment that promotes osteoprogenitor migration and subsequent ossification (Bixel et al., 2024). Compromised vascularization significantly impairs the healing of critical-sized calvarial defects (Caliaperoumal et al., 2018).

Aging bone tissues are inherently more hostile and pro-apoptotic(Almeida, 2012), leading to limited success with cell-based repair approaches in aged individuals(Hirata et al., 2022; Liu et al., 2018). Stiffness increases with aging in various tissues(Ferrari and Pesce, 2021; Lim and Ito, 2022; Segel et al., 2019), impacting cytoskeletal configuration(Khan et al., 2020; Pongkitwitoon et al., 2016) and significantly affecting osteogenic differentiation and senescence *in vitro*(Liu et al., 2022). Our study shows increased actin expression and elongated nuclear shape in aging osteogenic compartments, alongside elevated levels of Mmp9 and Mmp14, linked to stiffer osteogenic tissues(Tang et al., 2013; Zhu et al., 2020). These changes are associated with a decline in both the number and function of osteoprogenitors, including impaired proliferation, mitochondrial energy metabolism, Wnt/β-catenin pathway activation, migration, and ultimately, bone defect repair. While Wnt-bandage treatment increases the number of osteoprogenitors in the defect site across all age cohorts, it only modestly enhances bone repair in aged animals compared to the healing observed in adult mice.

We show that interventions improving whole-body energy metabolism can enhance aged bone health and repair. While IF is effective in delaying aging in adult flies, animals need to switch back to *ad libitum* feeding at older ages(Catterson et al., 2018), and increased protein intake is suggested for optimizing health span and longevity in aged mice and humans(Levine et al., 2014). Our findings, however, reveal a distinct paradigm for IF in aged bone repair. Specifically, short-term IF initiated post-injury robustly enhances bone healing in aged animals, comparable to younger counterparts.

Mechanistically, IF exerts its effects through several pathways, including enhanced stress resistance, increased reliance on lipid fuel utilization, and activation of proteostatic mechanisms, all contributing to delayed aging(Longo and Anderson, 2022). Although we did not observe distinct changes in overall signature of lipid metabolism or glycolysis in osteogenic tissues during aging or IF, we did find a decline in mitochondrial function and ATP supply with age. Our *in vitro* studies showed that alternating cycles of serum deprivation and supply in osteogenic cells induced genes related to the unfolded mitochondrial protein response, mitophagy and mitochondrial fission associated with increased mitochondrial membrane potential and mitochondrial fission, indicating improved function(McCarron et al., 2013; Westermann, 2012).

Our transcriptomic analysis shows that Mmp14 is reduced during IF in the periosteum. In the suture mesenchyme, Piezo1, a gene known to mediate mechanically induced aging(Ren et al., 2022; Segel et al., 2019), was also significantly reduced in aged mice undergoing IF. Along with the reversal of nuclear cell morphology and levels of F-actin, these results indicate that IF also impacts the mechanics of the tissue, making it resemble that of younger animals. IF also upregulated Dbp, Tef, and Hlf, genes involved in the circadian transcriptional network (Gachon et al., 2004), in both osteoprogenitor compartments of aged mice. These genes, which act on D-box sites, are linked to premature aging when knocked out, affecting the liver, kidney (Gachon et al., 2006), and hematopoietic stem cell differentiation. While circadian rhythms' impact on bone is mostly studied in osteoblasts *in vitro*(Luo et al., 2021; Zhou et al., 2018), our data suggest that circadian transcription may play a role in rejuvenating $CD90^{+}$ cells for bone formation and repair in aged mice. ChIP-SEQ studies (Yoshitane et al., 2019) have identified D-box sites near promoters of Wnt/β-catenin components (e.g., Lrp5/6, Wnt8a, Fzd4) and osteogenic transcription factors like Zbtb16, linking circadian rhythms, stem cell function, and Wnt signaling (Lecarpentier et al., 2014; Matsu-Ura et al., 2018). While food intake regulates circadian rhythms in several organs (Damiola et al., 2000; Pickel and Sung, 2020), the diet-circadian interaction in bone repair is not well understood. Investigating circadian transcription's role in enhancing osteoprogenitor function for bone repair in aged mice is promising.

IF also led to beneficial shifts in gut microbiota composition, including elevated levels of *Akkermansia muciniphila* and increased expression of genes involved in NAD^{+} biosynthesis. Subsequently, we show that short-term supplementation with *Akkermansia muciniphila* or NMN in aged mice significantly improve periosteal blood vessel density, osteoprogenitor number and function, osteoclast activity and enhance bone health and repair.

Although IF, Akk or NMN can act through many pathways and cellular processes, previous studies highlighted their beneficial effect on metabolism particularly their influence on NAD-dependent pathways. Mechanistically, our *in vitro* studies using calvarial osteogenic cells revealed that NMN protects against mitochondrial damage, often associated with aging, promotes healthy mitochondrial morphology and function, and enhances osteogenesis.

The effects of the three metabolic approaches on bone health and repair have not been studied in the context of already-aged individuals. NMN shows protective effects against bone health decline, but in long-term studies that begin dosing well before old age(Kim et al., 2021; Mills et al., 2016; Song et al., 2019). The impact of *Akkermansia muciniphila* on bone health or healing has only been investigated in young adult models with subcritical defects(Lawenius et al., 2020; Liu

et al., 2020). IF diet has been investigated for skin wounding(Luo et al., 2020) in young animals but not for bone repair. We present key progress in developing three clinically relevant approaches for bone tissue regeneration in elderly patients. First, we describe the effects of NMN or *Akkermansia* dosing, or IF diet on bone tissues in aged animals that have already experienced severe skeletal decline. Second, our rejuvenation protocols are applied for shorter terms, and even post-injury with IF diet.

This study offers valuable insights into bone aging and rejuvenation strategies. However, future research should address several limitations. Further investigation is needed on the mechanisms of action of IF, NMN, and *Akkermansia*, optimal intervention timing and duration, and synergistic effects of combined approaches. While this study focused on a common osteoprogenitor, investigating the roles of specific skeletal stem cell populations and other cells within the osteogenic compartments (including immune cells) in flat bones represents a compelling frontier.

Our findings suggest broader implications beyond bone health, with these rejuvenation strategies potentially translating to other aging organs and injuries. Rigorous investigation of dosage, safety, and long-term effects will be essential for human applications.

Materials and Methods

Wnt3a bandage manufacture

Polycaprolactone (PCL)-based polymer film (Poly-Med) was plasma-treated using a plasma etching electrode in oxygen gas at a flow of 20 cm^3 min^{-1} with a radio frequency plasma generator (frequency 13.56 MHz, power 50 W, Diener electronic Zepto-W6) at 0.2 mbar pressure for 3 min. Treated films were immediately incubated in 5% (3-Aminopropyl)triethoxysilane (APTES, Sigma, 440140) in ethanol (dark, room temperature, 2 hours), before washing twice with 100% ethanol (room temperature, 10 minutes), and allowed to air dry. Circular bandages were cut using a 5mm biopsy punch (Stiefel) and placed in 10 mg/ml succinic anhydride (Sigma Aldrich, 239690-50G) in 0.4M borate buffer (Sigma Aldrich, B7660) pH=9 (room temperature, 30 minutes). Bandages were twice washed in 25mM Morpholineethanesulfonic acid (MES) buffer pH 5 (Sigma Aldrich, M3671), then incubated in a MES buffer solution containing 50 mg/ml N(3Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma Aldrich, E7750) and 50 mg/ml NHydroxysuccinimide (NHS, Sigma Aldrich, 56480) (room temperature, 30 minutes), and washed twice again in MES buffer. Bandages are individually placed on a sterile flat surface and briefly allowed to air-dry. 300ng Wnt3a (R&D, 1324-WN-010) was diluted to 23 µL in MES buffer, and this liquid added as a droplet on top of the bandage for 1 hour at room temperature.

For inert control bandages (inactive-bandages), the bandages were then placed into a 96 well plate and washed with PBS. 20 mM Dithiothreitol (DTT, Roche, 10197777001) in 50 µL ddH_2O was then added and incubated (37°C, 30 minutes). Active Wnt3a-bandages were also placed in the 96 well plate, and then all bandages were washed with PBS, and quenched with 2% Fetal Bovine Serum (FBS, Sigma Aldrich, F7524) in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, 41966) (room temperature, 15 minutes) in preparation for their surgical implantation.

Wnt3a-bandage testing

The Wnt/β-catenin pathway activation of Wnt3a-bandages was confirmed as previously published (Lowndes et al. 2017). Wnt3a-bandages were tested for Wnt/β-catenin pathway activation using Comma-D Beta cells harboring GFP expression under the control of a TCF-LEF promoter (Lowndes et al. 2016) (**Figure 1 – figure supplement 3a, c**). Similarly, LS/L cells harboring luciferase under control of a 7xTCF promoter were used to quantitatively test Wnt3a-bandages, as described previously (Okuchi et al. 2021) (**Figure 1 – figure supplement 3a, c**).

Animal experimentation methods according to the ARRIVE Essential 10 guidelines

All procedures involving animals were carried out by holders of Personal Licences (PIL) under a Project Licence (PPL number: P8F273FDD, S.J.H.) shaped by ARRIVE and NC3Rs guidelines. The PPL was approved by the Animal Welfare and Ethical Review Body (AWERB) at King's College London and granted by the Home Office under the Animals (Scientific) Procedures Act 1986 (ASPA) and Amendment Regulations 2012.

Study design

Within each animal, the bone repair under the Wnt-bandage was compared to the one under the inactive bandage wherever appliable. Between groups of treatments, the bone repair was compared to other groups, or to a control group (sham treatment/ad libitum feeding). Each animal was used as an experimental unit.

Sample size

10 animals were used per surgical group for a total of 60 animals. The minimum is 7 animals/group to get statistically reliable tests of the hypothesis (80% power with 0.05 type I error). We applied the resource equation method (Charan and Kantharia, 2013). Based on our experience 8-10 animals were used per group, to account for morbidity and animal variability (Okuchi et al., Nature Materials 2021).

Inclusion and exclusion criteria

All animals were included in the study. Animals that were scarified/found dead before the end of the study were excluded (two for the group "Adult" (n=8), and one for the groups "Aged" (n=9) and "Aged + Akk." (n=9), n=10 in all other groups).

Randomization

Randomization method was not applied to assign animals to experimental groups. Animals were identified using ear notches.

Blinding

The experimenter was aware of the group allocation of the animals when performing the experiments and during the data acquisition and analysis.

Outcome measures

Bone repair was assessed using microCT as described in the Micro-Computed Tomography section, and subsequent histological analysis and immunology staining were performed on cryosections as described in the Sample Processing and Cryosectionning, and Fluorescence Immunostaining sections.

Experimental animals

Female mice were chosen for their delayed healing and higher fracture risk (Haffner-Luntzer et al., 2021; Ortona et al., 2023), leading to a more challenging case for bone repair, making them a stringent test for evaluating the effectiveness of our rejuvenation approaches. Female C57BL6/J mice (Charles River, strain 632) were housed in conditions that comply with the Animals (Scientific) Procedures Act 1986 (ASPA). Mice were group housed (2-5 mice/cage) under 7 a.m.-7 p.m. lighting and fed with standard mouse food pellets (LabDiet, 5053). Three age groups were used for this study: Young (6 weeks), Adult (13 weeks), and Aged mice (> 88 weeks).

For *ad libitum* diet, the food was renewed every 3 to 4 days. During intermittent fasting, food was removed from cages at 10 a.m. on Monday, Wednesday, and Friday, and returned at 10 a.m. on Tuesday, Thursday, and Saturday, such that the maximum time without food was 24 hours. Intermittent fasting started the day after the surgery and was performed for 10 weeks.

Caloric intake calculation

To assess the caloric intake of mice, the food was weighted when made available to the mice (W_{in}), and when removed (W_{out}). The daily consumed food was calculated based on the weight difference (W_{in} - W_{out}), then converted to kcal (1 g = 3.02 kcal, LabDiet, 5053), and expressed as kcal/mouse/day for each cage (n cage ≥ 3 with 1 to 5 mice/cage).

Blood glucose level measurement

Fasting blood glucose levels were measured (Accu-Check tests strips) from 6h fasting mice by blood sampling the tail vein. For intraperitoneal glucose tolerance test (IPGTT), glucose was injected intraperitoneally (2 g/kg), and the blood glucose levels were measured after 15, 30, 60 and 120 minutes.

Experimental procedures

Calvarial Defect

All steps of surgery were carried out under aseptic conditions. Mice were intraperitoneally anaesthetized with 10 µL/g bodyweight of anesthesia cocktail (7.5 mg/ml Ketamine hydrochloride (Ketavet) and 0.1 mg/ml Medetomidine hydrochloride (Domitor) in 0.9% sterile saline). Loss of consciousness was confirmed with lack of pedal and tail reflexes. The top of the head was shaved, cleaned, and prepared for surgery, before a ~8 mm central incision was made in the skin with surgical scissors. The periosteum was gently pushed towards the outer edges of the calvaria, and the location of the sutures noted. A 2 mm diameter full thickness defect was drilled into the middle of the parietal bone on both sides of the skull, ensuring irrigation was applied to limit heat buildup. A small amount (~10 µL) of 1 mg/ml collagen gel (Corning, 354249) (diluted with DMEM and set with 3 mM sodium hydroxide (Sigma Aldrich, S5881)) was placed into the defect to exclude trapped air, and the required bandages were placed face down over the defect. Small dots of glue (3M, Vetbond) were applied at the edges of the bandages to affix them to the surface of the skull. The skin was then approximated and glued to ensure closure of the wound. Mice were administered 10 µL/g bodyweight of the recovery cocktail intraperitoneally (0.125 mg/ml Atipamezole hydrochloride (Antisedan) and 0.01 mg/ml Buprenorphine hydrochloride (Vetergesic) in 0.9% sterile saline) and placed in a warmed cage (35 °C). Mice typically became conscious within 20 minutes, began moving around the cage within 40 minutes, and were feeding within 2 hours. Mice showed no signs of distress or pain the following day and were externally monitored daily for the healing of the skin wound. Following surgery, mice were maintained under standard conditions for 10 weeks, and group housed (2-5 mice/cage) where possible.

NMN supplementation

600 mg/kg bodyweight doses of β-nicotinamide mononucleotide (NMN, APExBIO, B7878), dissolved in water were given by flexible-tipped oral gavage 3 times per week, not on consecutive days, from 2 weeks before surgery to 2 weeks after, a total of 4 weeks.

Anaerobic Culture of Akkermansia muciniphila

Preserved *Akkermansia muciniphila* (Collection de l'Institut Pasteur, CIP107961T) was resuspended and cultured in Gifu's Anaerobic Medium (GAM) broth (Nissui Pharmaceutical, 05422) in an anaerobic environment (80% Nitrogen, 10% Carbon dioxide, 10% Hydrogen). Aliquots were

cryopreserved in 50% glycerol (Sigma Aldrich, G2025) in PBS. Post-thawing viability was determined by serial dilution of cryopreserved aliquots, plating onto GAM-Agar (Sigma Aldrich, 01916) plates, and 24-hour anaerobic culture.

Akkermansia muciniphila supplementation

Cryopreserved doses of 2.0 x 10^8 C.F.U. *Akkermansia muciniphila* were warmed to ~ 30°C and administered to Aged mice by flexible-tipped oral gavage 3 times per week, not on consecutive days, from 2 weeks before surgery to 6 weeks after, a total of 8 weeks.

Results

Results are displayed as mean ± SD. All numerical values are available in the Source Data files.

Ethical Approval for animal experimentation

All procedures involving animals were carried out by holders of Personal Licences (PIL) under a Project Licence (PPL, P8F273FDD, S.J.H.) shaped by ARRIVE and NC3Rs guidelines. The PPL was approved by the Animal Welfare and Ethical Review Body (AWERB) at King's College London and granted by the Home Office under the Animals (Scientific) Procedures Act 1986 (ASPA) and Amendment Regulations 2012.

Microbiome Profiling

Feces samples were freshly collected and snap-frozen in liquid nitrogen. Samples were submitted for full length (V1-V9) 16S metagenomic profiling. Amplification with KAPA HiFi HotStart ReadyMix PCR Kit (KAPA) and 16S barcode primers (PacBio) preceded library preparation SMRTbell Express Template Prep 2.0 (PacBio). Samples were sequenced via the Illumina MiSeq to generate amplicon sequence data specifying the V3-V4 regions of the 16S rRNA ribosomal subunit. The metagenomic analysis was conducted using the amplicon sequence variant (ASV) method of taxonomic classification facilitated by DADA2 (Callahan et al. 2016) incorporated by the Qiime2 package (Bolyen et al. 2019). Quality control metrics of the amplicon data was visualized by FastQC (Andrews S. 2015). The data was cleaned, via removal of adapters and low-quality sequences, using Trimmomatic (Bolger et al. (2014) and Qiime2.

Micro-Computed Tomography

Mice were culled by cervical dislocation and their tissues immediately collected. Whole calvarial samples were fixed in 4% PFA (room temperature, overnight) and washed in PBS. Fixed samples were scanned on a µCT50 micro-CT scanner (Scanco) in 19 mm scanning tubes, using voxels of side length 10 µm, X-ray settings of 70 kVp and 114 µA and using a 0.5 mm aluminum filter to attenuate harder X-rays. Scans were reconstructed and calibrated using known densities of hydroxyapatite (HA) between 0 and 790 mg HA cm^{-3}, with absorption values expressed in Hounsfield Units (HU). 3D volumes were constructed from raw data in SCANCO Visualizer 1.1, and signal of less than 250 mg HA cm^{-3} was considered non-mineralized and was set as the black level in displayed images.

Sample Processing and Cryosectionning

Fixed samples were decalcified in 10% formic acid (room temperature, overnight), then washed in PBS. Dehydration was carried out in 30% sucrose/PBS (room temperature, overnight), followed by 30% sucrose/30% OCT/PBS (room temperature, overnight). Samples were embedded in 100% OCT solidified with dry ice and stored at −80°C. Cryosections were cut at 14 µm thickness using a cryostat microtome at −23°C. Cut sections were stored at −20°C.

Fluorescence Immunostaining

Sections were rehydrated to ddH_2O. Antigen retrieval was performed with 0.05% v/v Tween-20 (Sigma Aldrich, P9416) in 10 mM Tris (Sigma Aldrich, 93362), 1 mM EDTA (Sigma Aldrich, E6758), pH=9.0 (40°C, 30 minutes). Sections were then permeabilized with 0.1% Triton X-100 (Sigma Aldrich, 93443) in 1% Bovine Serum Albumin (BSA, Sigma Aldrich, A2153) in phosphate buffered saline (PBS, Sigma Aldrich, P4417) (1% BSA/PBS) (room temperature, 5 minutes), washed with 1% BSA/PBS, and individual staining regions were designated with hydrophobic delimiting pen (Agilent, S200230-2).

Primary antibodies were diluted in 0.05% Triton-X in 1% BSA/PBS and incubated in a humid chamber (4°C, overnight). Triplicate washes of 0.1% Triton-X in 1% BSA/PBS were carried out (room temperature, 10 minutes). Secondary antibodies were diluted in 0.05% Triton-X in 1% BSA/PBS, with 5 µg/ml DAPI (Sigma Aldrich, D9542), and 1:400 dilution Phalloidin-488 (Thermo Fisher, A12379) and incubated in a humid chamber (room temperature, 1 hour). Triplicate washes of 0.1% Triton-X in 1% BSA/PBS were carried out (room temperature, 10 minutes) before mounting with aqueous mounting media (Abcam, ab128982). Immunostained sections were imaged with an inverted spinning disk confocal microscope (Nikon, NISElements Viewer 5.2 software). Optimal z-axis step size was determined according to the Nyquist criteria for sampling. Images were post-processed and analyzed through ImageJ/FIJI.

Primary antibodies

Anti-Endomucin (Emcn) 1:100 (Santa Cruz, sc-65495)

Anti-CD90/Thy1, 1:100 (Abcam, ab3105)

Anti-phosphoAMPK (Thr183, Thr172), 1:100 (Thermo Fisher, 44-1150G)

Anti-ATPB, 1:100 (Abcam, ab128743)

Anti-Ki67, 1:100 (Thermo Fisher, MA5-14520)

Anti-p16INK4a, 1:100 (Abcam, ab211542)

Anti-beta-catenin 1:100 (Abcam, ab32572)

Anti-MMP2, 1:100 (Abcam ab92536)

Anti-CD31, 1:100 (Novus Biologicals, NB100-2284)

Anti-Lamin B1, 1:100 (Abcam, ab16048)

Secondary antibodies

Chicken anti-Rat-647 1:1000 (Thermo Fisher, A21472)

Donkey anti-Rabbit-555 1:1000 (Thermo Fisher, A32816)

Fluorescence thresholding quantification

See **Figure 1 - figure supplement 1e**. Sample tissue sections were immunostained alongside controls that lacked primary antibody: the 'no primary control'. These control samples were imaged using the same microscope settings as the experimental samples, and maximum projections were calculated to reduce volumetric images to 2D. The maximum pixel intensity in

the control images was used to threshold the experimental samples, thereby excluding signal arising from non-specific staining by fluorescent secondary antibodies. Cellular localization of the remaining signal allowed designation of cells positive and negative for the antibody targets of interest.

For quantitative measurements of F-actin/Phalloidin-488 signal intensity (e.g. **Figure 1h**), sum-projections were taken to reduce volumetric 3D images to 2D space. In these quantifications, mean sum pixel intensity was measured and the mean sum background pixel intensity subtracted for N ≥ 56 cells in n = 3 mice.

Tetramethylrhodamine (TMRM) & Hoechst staining in live tissue *ex vivo*

Periosteum was dissected from n = 3 freshly killed mice, and immediately placed in warmed culture media (10% FBS in DMEM) containing 100 nM TMRM (Thermo Fisher, I34361) and 5 µg/mL Hoechst 33342 (Thermo Fisher, H3570) for 30 minutes at 37°C. Tissue was briefly washed with fresh warmed media and imaged on a Zeiss widefield microscope (Zeiss Zen Blue Edition 2.5 software). Explanted cell controls were allowed to migrate from minced suture mesenchyme and attach for 24 hours before identical TMRM/Hoechst staining.

MC3T3 cell culture

MC3T3 (gift from Sophie Verrier, AO foundation, Davos, Switzerland) cells were cultured in MEMα (Thermofisher, A1049001) 10% foetal bovine serum (FBS; Thermofisher A5256701), 1% penicillin-streptomycin (PS; Thermofisher, 15140-122). Cells were used at passages 13-20 and seeded at 25,000 cells / cm^2 for further experiments in glucose- and pyruvate-free DMEM (Thermofisher, 11966025), 10% FBS, 1% PS. All experiments were conducted in differentiation medium, consisting of glucose- and pyruvate-free DMEM 10% FBS, 1% PS, 50 µM ascorbic acid (Merck, 5960), and 2 mM glycerophosphate (Merck, 50020). Alternate serum deprivation was performed by replacing the osteogenic media by serum-free osteogenic media every 1h for 6h. For oligomycin and NMN treatments, cells were treated by 0.1 µM oligomycin (Merck, 75351), with or without 1 mM ý-nicotinamide mononucleotide (NMN; APExBio, B7878), or with 0.1% DMSO (Merck, D2438), the vehicle of oligomycin. Cells were treated for 6h before switching to differentiation medium ("oligomycin" or "DMSO" condition) or differentiation medium + 1 mM NMN ("oligomycin + NMN" condition). The media were changed twice a day.

Inhibition of ATP production in MC3T3 and measure of Reactive Oxygen Species (ROS) production

ATP level in cells was measured in 96 well plates after 6h of the treatment induction, using the CellTiter-Glo® 2.0 Assay (Promega, G9241) according to manufacturer's instructions. ROS production was measured in live cells using the CellROX deep red staining kit (Thermofisher, C10422) using a spinning disk microscope (Nikon Ti2, Crest Optics X-Light V3), with the sum projection intensity normalized by the number of cells using Hoechst staining.

MC3T3 count and mitochondrial activity

Cell count and mitochondrial activity were measured with Hoechst and TMRM staining respectively, using a spinning disk microscope and according to manufacturer's instructions. Z-stacks of 20 µm were acquired and the TMRM sum intensity was normalized to the cell number.

Holographic imaging

Mitochondrial morphology was observed using holotomography imaging on live cells (Tomocube, HT-X1), with a reference refractive index set at 1.337. After reconstruction of the images, mitochondria were categorized in three types according to their shape. The proportion of each morphology type was determined with Tomo Analysis and Fiji software. MitotrackerGreen (Thermofisher, M7514) was used to stain mitochondria and confirm the mitochondrial morphology obtained by holotomography.

RT-qPCR

RNA was extracted according to manufacturer's instructions (Marchery-Nagel, M36006), 12h after the treatment induction. Reverse transcription (Thermofisher, 4374966) and qPCR (Thermofisher, 4368706) were performed according to manufacturer's instruction and mRNA target levels were normalized by beta-Actin mRNA levels using the $2^{-ýýCt}$ method. The following primers have been used (5'è 3'): *MFN1*: F-AACTTGATCGAATAGCATCCGAG, R-GCATTGCATTGATGACAGAGC; *MFN2*: F-CTGGGGACCGGATCTTCTTC, R-CTGCCTCTCGAAATTCTGAAACT; *DRP1*: F-GGGCACTTAAATTGGGCTCC, R-TGTATTCTGTTGGCGTGGAAC; *PGC1a*: F-AGTGGTGTAGCGACCAATCG, R-AATGAGGGCAATCCGTCTTCA; *ARBP*: F-AGATTCGGGATATGCTGTTGG, R-AAAGCCTGGAAGAAGGAGGTC; *PINK1*: F-CACACTGTTCCTCGTTATGAAGA, R-TTGAGATCCCGATGGGCAAT; *PARKIN*: F-GAGGTCCAGCAGTTAAACCCA; R-CACACTGAACTCGGAGCTTTC; *LC3II* F-TTATAGAGCGATACAAGGGGGAG, R-CGCCGTCTGATTATCTTGATGAG; *ATG5*: F-AAGTCTGTCCTTCCGCAGTC, R-TGAAGAAAGTTATCTGGGTAGCTCA; *LONP1*: F-ATGACCGTCCCGGATGTGT, R-CCTCCACGATCTTGATAAAGCG; *HSP60*: F-CACAGTCCTTCGCCAGATGAG, R-CTACACCTTGAAGCATTAAGGCT; *HPSA9*: FAATGAGAGCGCTCCTTGCTG, R-CTGTTCCCCAGTGCCAGAAC; MTCO1: F-TATGTTCTATCAATGGGAGC, R-GTAGTCTGAGTAGCGTCGTG; beta-Actin: F-GGCTGTATTCCCCTCCATCG, R-CCAGTTGGTAACAATGCCATGT.

Evaluation of the osteoblastic differentiation

Cells were cultured for 2 weeks before the staining of the alkaline phosphatase (ALP; Abcam, ab284936) or mineralized matrix using alizarin red S (ARS; 2% solution in H_2O) (Merck, A5533). ALP staining was performed as per manufacturer's recommendation, and the stained area were quantified using Fiji on pictures taken under a Evos XL core microscope. For ARS staining the cells were fixed with 4% paraformaldehyde in PBS, stained by ARS then imaged using a Evos XL core microscope. The stained mineralized matrix was then dissolved with 10% (v/v) acetic acid in H_2O and optical density was measured at 405 nm to quantify the amount of dissolved ARS.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9. All data are plotted as mean, with error bars indicating standard deviation. Typically, experimental data was analyzed from all mouse conditions together using one-way ANOVA, with individual comparisons between conditions/regions of interest displayed on figures as Welch's two-tailed unpaired tests. Welch's ANOVA was used instead of ordinary ANOVA because it is more conservative and reduces the risk of false-positive p-values. Two-factors comparisons were analyzed with two-way ANOVA with uncorrected Fisher's LSD test. Only the statistical values for the hypotheses being tested are shown; multiple comparison corrections were not applied. Ratio-paired two-tailed t-test was used in comparisons within the same mouse (i.e., comparisons of Wnt3a-bandage vs Inactive-bandage). Correction of multiple comparison was not applied as each p-value was considered for itself. On some tested groups, correction for multiple comparison did not change our general conclusions. Uncorrected Dunn's tests were performed for the statistical analysis of UCell gene-expression signature scoring, as a non-parametric test for non-Gaussian distributions of data.

RNAseq sample processing

Periosteum and suture mesenchyme were separately dissected and pooled from ≥ 3 live mice from each condition, briefly minced with a scalpel blade, and immediately placed in 250 µL of a solution of 0.5 mg/ml Liberase TL (Roche, 05401020001) and 0.1 mg/ml DNaseI (Sigma Aldrich, DN25) in Hanks' Balanced Salt Solution (HBSS, Gibco, 14170-120) and incubated for 25 minutes at 37 °C with shaking (600 rpm). The digestion was quenched with ice-cold 1mL buffer (3% FBS in PBS), passed through a 100 µm cell strainer (Corning, 352360), and centrifuged (500 g, 5 minutes, 4°C). The supernatant was removed, and the cell pellet gently resuspended in staining solution containing 0.1 µg 'hashtag' antibody, 50 µM DR (Invitrogen, 65-0880-92), and 1:4000 DAPI in ice-cold 3% FBS/PBS. Up to 4 samples were individually stained with TotalSeq™ antibodies 1-4 (Biolegend 155801, 155803, 155805, 155807). Samples were centrifuged, washed with 3% FBS/PBS, centrifuged again and resuspended in 3% FBS/PBS for single-cell FACS. The gating strategy took single (by forward and side scatter), nucleated ($DRAQ^{+}$), live ($DAPI^{-}$) cells, and up to 4 individually-hashtagged samples were multiplexed together by sorting equal cell numbers into the same collection tube, and stored on ice to preserve viability. Cells were loaded onto a 10X Chromium controllers and the library generated through single cell 10X 3' v3.1 gene expression. All libraries were sequenced together using NextSeq 2000 sequencing on a P3 flow cell.

RNAseq data processing

Initial alignment and processing were performed through the CellRanger pipeline. Further quality control, processing, and analysis was carried out through the Seurat (v4) package (Hao et al. 2021) and additional packages in R software (R Core Team (2022), v4.2.1). Cells were graded for proportion of mitochondrial genes, and this metric regressed. The immune cell $CD45^{+}$ populations in the samples ($Ptprc^{high}$) were removed from downstream analyses. $CD90^{+}/CD45^{-}$ ($Thy1^{high}/Ptprc^{low}$) clusters were considered the populations of interest. Differential expression of lincRNA Gm42418 was identified as an artefact and removed from the data, as has been done in other studies (Kimmel et al. 2019). For calculation of Gene Ontology (GO) and KEGG enrichment of sets of differentially expressed genes, ShinyGO 0.76.3 (Ge, Jung, and Yao 2020) was used to calculate false discovery rate (FDR) and fold enrichment (FE) values. Full details of differentially expressed genes in comparisons between ages, diets, and $CD90^{+}$ subpopulations are available in Source Data 2.

To construct the senescence signature, we obtained mouse aging-related genes from the GenAge database (*https://genomics.senescence.info/genes/search.php?organism=Mus+musculus&show=4*). The genes were categorized into two groups: positively regulated and negatively regulated. Positively regulated genes are defined as those with high expression levels associated with senescence, while negatively regulated genes are defined as those with low expression levels associated with senescence. The genes were clustered based on their expression, then we selected the highly expressed cluster in the positively-regulated genes and the poorly expressed cluster in negatively-regulated genes in the Aged group.

For the glycolysis and lipid catabolic processes signatures, we obtained mouse metabolism-related genes from AmiGO2 database (*https://amigo.geneontology.org/amigo*) with Ontology ID: GO:0061621 (glycolysis) and GO:0050996 (positive regulation of lipid catabolic process), and we clustered the genes based on their expression levels. Heatmaps were drawn by R package pheatmap (version 1.0.12).

Data availability

All numerical data included in the production of figures, plots and statistical analysis will be found in the Source Data. Additional statistical analysis values (F values, t values, degrees of freedom and p values) will be presented in the Source Data documents. Source Data will be provided with this paper. Raw and processed single cell RNAseq data will be available under a GEO accession number during the revision/review process.

Additional information

Author Contributions

S.J.H. conceived the project and with J.R. designed the experiments. J.R. performed surgeries, *ex vivo* experiments, *in vitro* experiments, and collected all data. J.R. analyzed microCT data, and live *ex vivo* staining J.R and P.B analyzed histological staining. P.T and P.B performed experiments in **Figure 6** and **7**. R.C. processed the metagenomic data. J.R., Y.T, A.V. and D.F. performed RNAseq analysis. J.R., P.T. and P.B. prepared the figures. J.R. and S.J.H. interpreted the data and wrote the initial manuscript draft. S.J.H revised the manuscript. All authors approved the manuscript. S.J.H. led the project and provided financial support for the project.

Competing interests

S.J. H is listed as an inventor on a patent application (P6125GB00) on Tissue Regeneration Patch that includes the Wnt-bandage.

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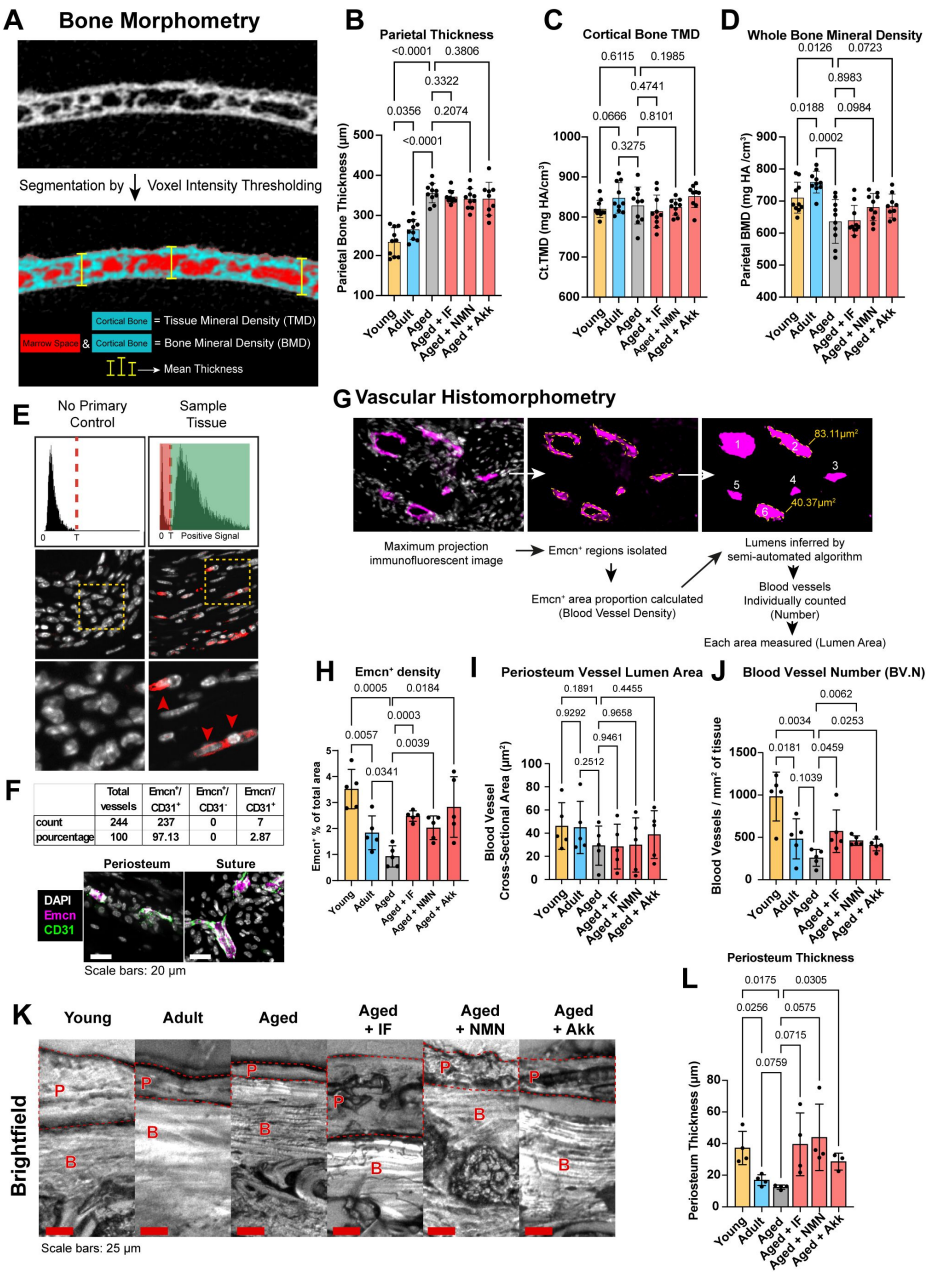


Figure 1 – figure supplement 1.

(**A**) Methodology for segmentation of micro-computed tomography (microCT) images for calculation of bone morphometry metrics: (**B**) Parietal Thickness (**C**) Cortical Tissue Mineral Density (Ct.TMD), (**D**) Parietal Whole Bone Mineral Density (BMD). Statistical analysis: Welch's tests, n = 10 mice per condition, except Aged + Akk (n = 9). (**E**) The method for quantifying immunofluorescence-positive (red) cells. The maximum pixel intensity is determined in control tissues lacking primary antibody (no primary control). This value is used to threshold the stained sample tissue, with the remaining signal distinguishing positive cells (red arrows). DAPI (grey) is also shown for reference of cellular locations. (**F**) Number and percentage of blood vessels positive for Emcn and/or CD31 with representative images in the periosteum and suture mesenchyme. (**G**) Methodology for segmentation of Endomucin (Emcn) immunofluorescent staining in fixed tissue sections, for calculation of vascular histomorphometry metrics: (**H**) Emcn$^+$ staining density (proportion of total tissue area). Data are shown previously in main **Figures 1**, 3, and 5, and are reproduced here for reference, (**I**) Mean lumen area per blood vessel, (**J**) Number of blood vessels per area tissue (BV.N). Statistical analysis: Welch's tests, n = 5 mice per condition. (**K**) Representative brightfield images of unstained tissue sections highlighting the overlying periosteum (red P, red dashed line) and parietal bone (red B). (**L**) Mean periosteal thickness. Statistical analysis: Welch's tests, n = 4 mice per condition, except Aged + Akk (n = 3).

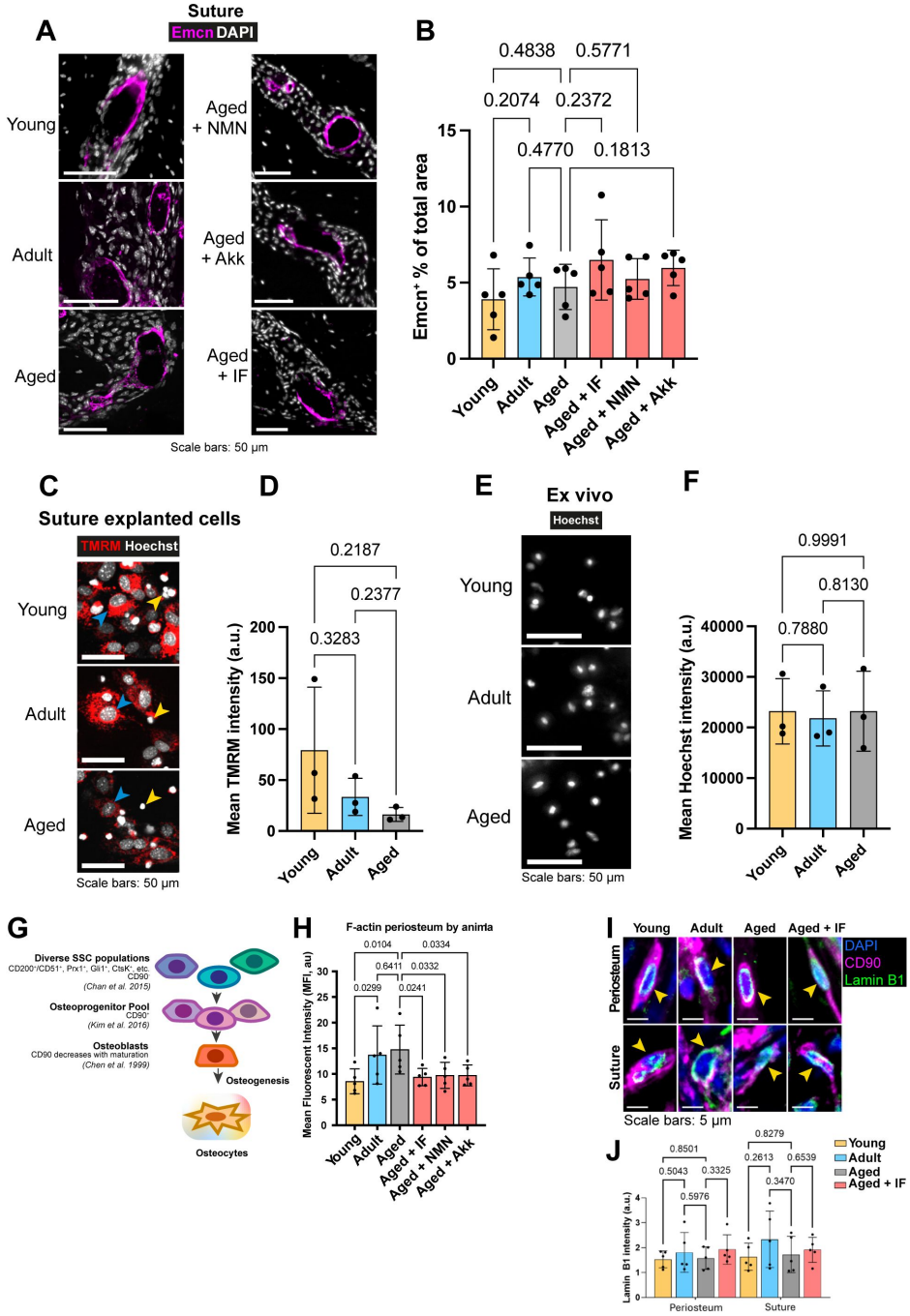


Figure 1 – figure supplement 2.

(**A**) representative immunofluorescence staining of Emcn (magenta) and DAPI (grey) and (**B**) mean cross sectional area of Endomucin (Emcn)-positive vascular cells in mouse sutures. Statistical analysis: Welch's tests in n = 5 mice. (**C**) Representative images and (**D**) mean intensity of live TMRM staining (red) and Hoechst (grey) in adherent cells allowed to explant from suture mesenchyme tissue for 24 hours *in vitro*. Remaining mesenchyme tissue was removed prior to staining. Statistical analysis: Welch's tests in tissue from n = 3 mice per condition. (**E**) Representative images and (**F**) mean intensity of live Hoechst staining (grey) from the TMRM experiment in **Fig. 1i-j**. Statistical analysis: Welch's tests in tissue from n = 3 mice per condition. (**G**) Schematic demonstrating the hierarchy and selected markers of Skeletal Stem Cells (SSCs), leading to the $CD90^+$ cell pool, which ultimately differentiates into osteoblasts and osteocytes during osteogenesis. (**H**) Mean Phalloidin-488 staining intensity for F-actin in the periosteum. Statistical analysis: Welch's tests in n = 5 mice. (**I**) Representative images depicting CD90 (magenta), DAPI (blue), and Lamin B1 (green). Yellow arrows indicate positive cells. (**J**) Normalized intensity staining for Lamin B1 in the nucleus of $CD90^+$ cells. Statistical analysis: Welch's tests in n = 5 mice per condition.

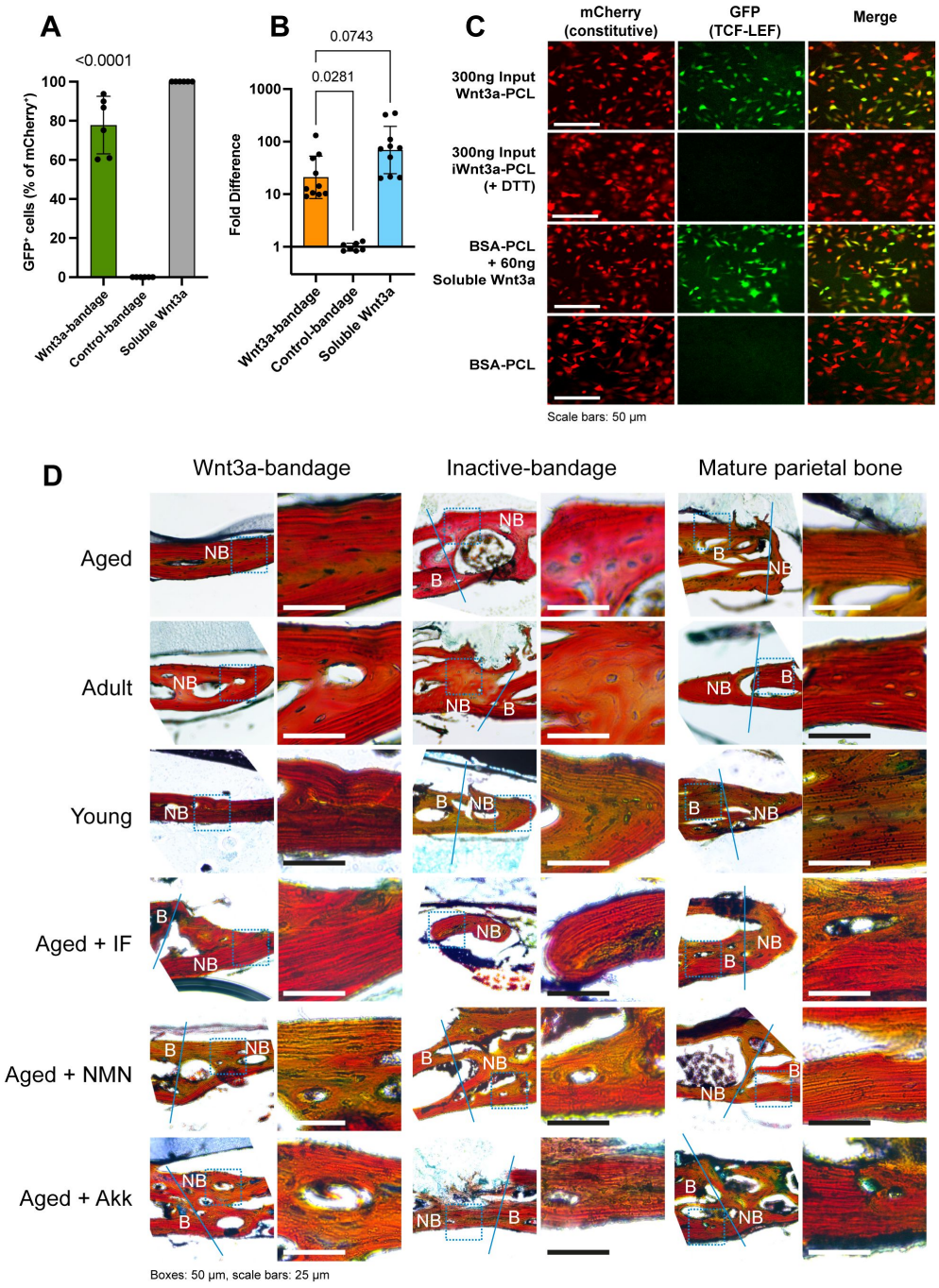


Figure 1 – figure supplement 3.

(**A**) Characterization of Wnt3a-bandage activation of the Wnt/β-catenin pathway with Comma-D Beta cells *in vitro*. Presented as the proportion of GFP$^+$ cells, normalized to the proportion of GFP$^+$ responsive to soluble Wnt3a. Statistical analysis: two-tailed one-sample t test against the inactive control-bandage (mean = 0); n = 6 for each condition. (**B**) Characterization of Wnt3a-bandage activation of the Wnt/β-catenin pathway with LS/L cells *in vitro*. Presented as fold change of Luciferase signal over control-bandage. Statistical analysis: Welch's test, n = 10 for each Wnt3a-bandage and Soluble Wnt3a, n = 7 for Control-bandage. (**C**) Representative images for Comma-D Beta cells quantified in (**A**). mCherry (red) is constitutively expressed, GFP (green) is expressed during Wnt/β-catenin pathway activation. Wnt3a is treated with DTT, resulting in complete chemical inactivation (iWnt3a). Bovine Serum Albumin (BSA) is used as an additional negative control. Polycaprolactone (PCL) is the polymeric material that Wnt3a is bound to during Wnt3abandage manufacture. (**D**) Representative Movat's pentachrome staining of bone tissues showing the lamellar/striated structures. Blue line designates the drilled defect edge, thereby separating original intact bone B from new bone NB. The dashed blue box shows the enlarged region.

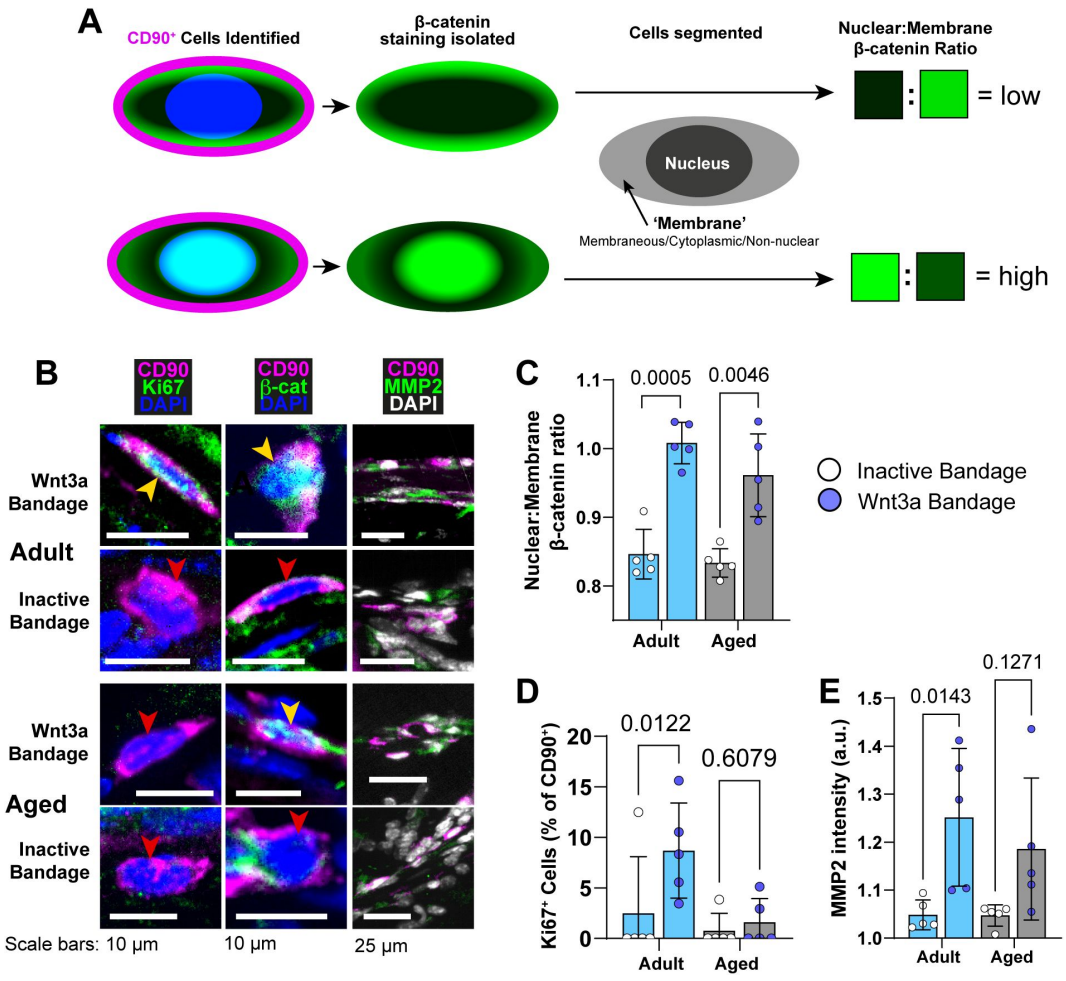


Figure 1 – figure supplement 4.

(**A**) Schematic depicting the methodology for measuring the ratio of Nuclear:Membrane β-catenin in single CD90$^+$ cells. (**B**) Representative images depicting CD90 (magenta), DAPI (grey/blue as indicated by column), and functional markers (green) as indicated by column). Yellow arrows indicate marker-high/positive cells; red arrows indicate marker-low/negative cells. (**C**) Proportion of CD90$^+$ cells that are Ki67$^+$ by age. Statistical testing: paired, two-tailed t tests in n = 5 mice per condition. (**D**) Ratio of Nuclear:Membrane β-catenin staining in CD90$^+$ cells by age. Statistical testing: ratio paired, two-tailed t tests in n = 5 mice per condition. (**E**) Normalized intensity staining for MMP2 in CD90$^+$ cells by age. Statistical testing: ratio paired, two tailed t tests in n = 5 mice per condition. For all plots (**C-E**), Wnt3a-bandage (dark blue points), inactive bandage (white points), Adult (blue), Aged (grey).

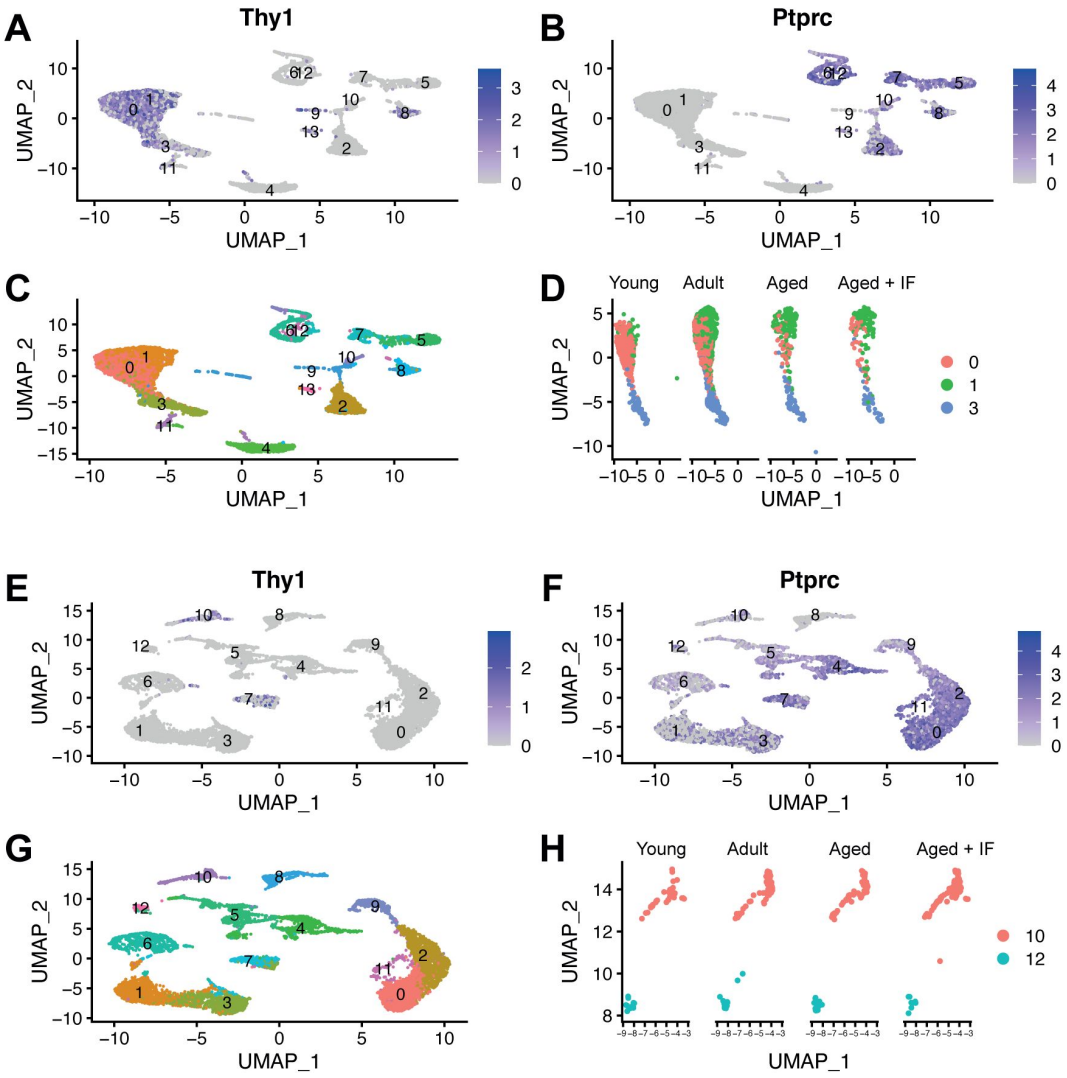


Figure 2 – figure supplement 1.

UMAP-coordinate plot of Thy1 (CD90, **A**) and Ptprc (CD45, **B**) expression in clusters of cells within the periosteum. (**C**) Periosteum cell populations (clusters) as identified through unbiased clustering (**D**) The $CD90^{+}/CD45^{-}$ clusters of interest (0, 1, 3), separated by mouse condition. UMAP-coordinate plot of Thy1 (CD90, **E**) and Ptprc (CD45, **F**) expression in clusters of cells within the suture. (**G**) Suture mesenchyme cell populations (clusters) as identified through unbiased clustering (**H**) The $CD90^{+}/CD45^{-}$ cluster of interest (10), and also the small endothelial cluster (12), separated by mouse condition.

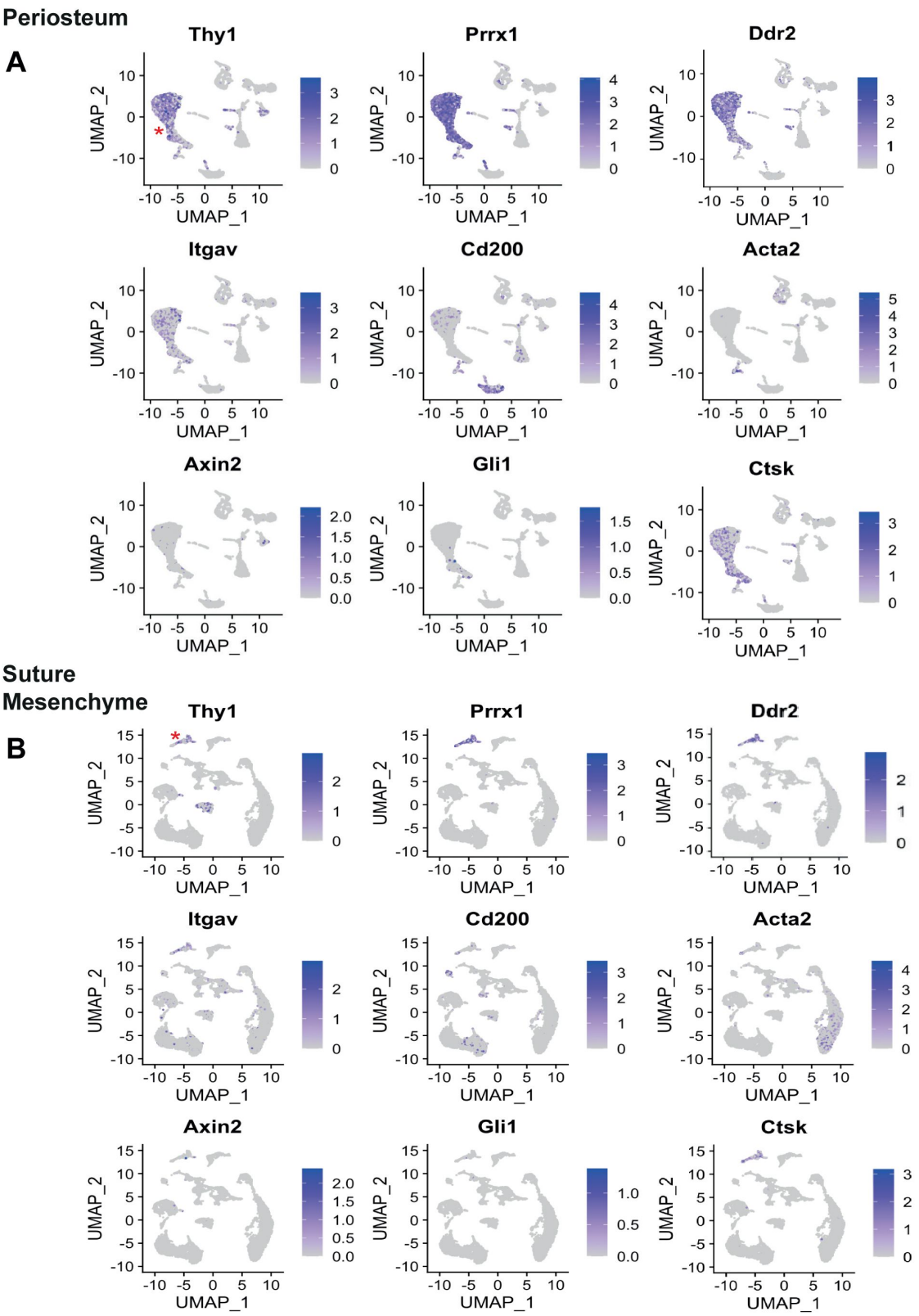


Figure 2 – figure supplement 2.

UMAP-coordinate plot of the entire cell populations from Periosteum (**A**) and Suture Mesenchyme (**B**). Expression of SSC markers (purple) highlights clusters of transcriptomically-similar cells. The red asterisk is adjacent to the group of cells referred to as the CD90^{+} (Thy1) population, which is also CD45^{-} (Ptprc^{-}) (**Figure 2 – figure supplement 1**). Prrx1 (Prx1), Ddr2, Itgav (CD51), Cd200 (CD200), Acta2(a-SMA), Axin2, Gli1 and Ctsk (Cathepsin K) are shown.

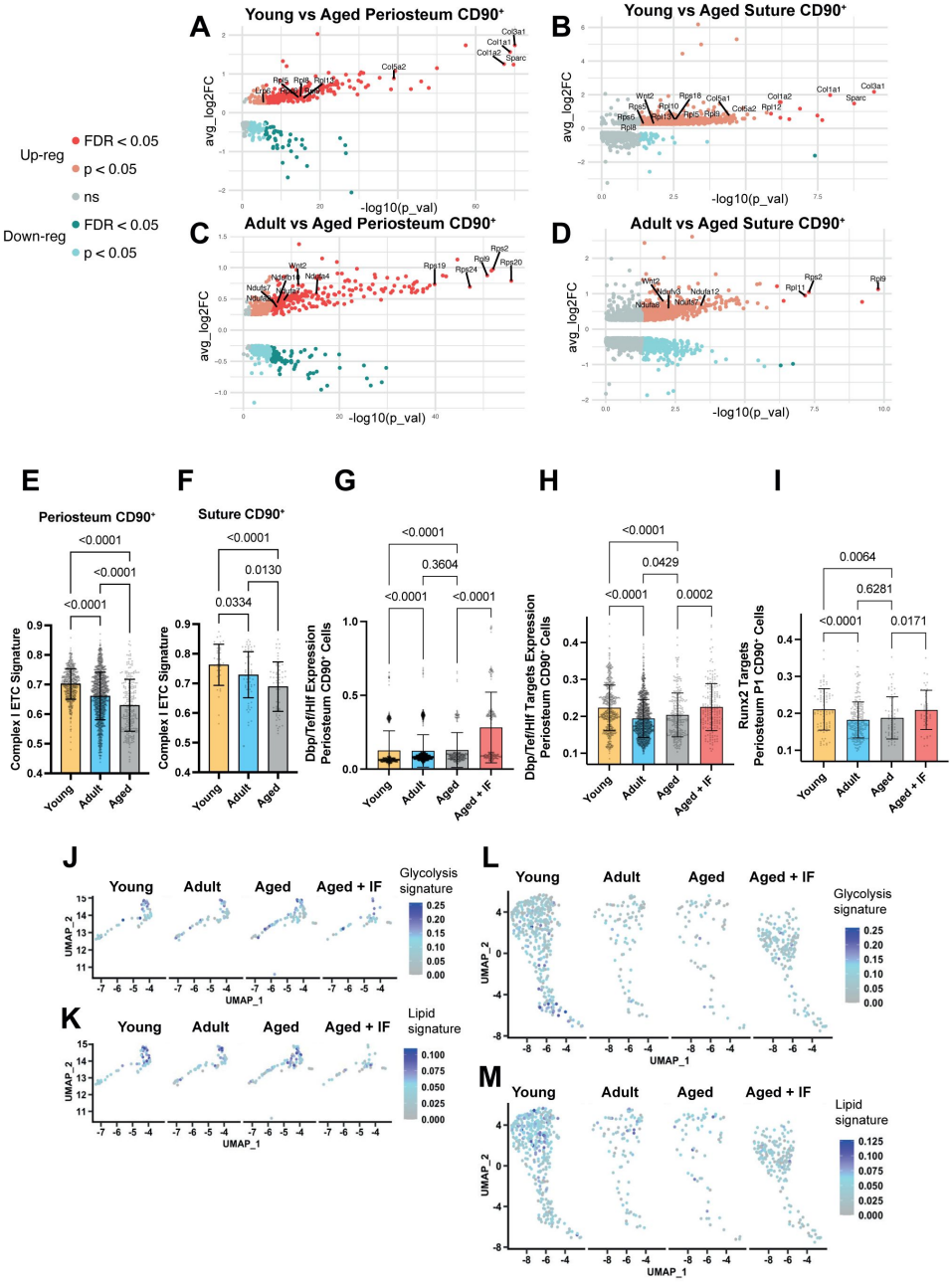


Figure 2 – figure supplement 3.

(**A-D**) Volcano plots showing differentially-expressed genes in the comparison of the CD90$^+$ population in (**A**) Young vs Aged periosteum; (**B**) Young vs Aged suture; (**C**) Adult vs Aged periosteum and (**D**) Adult vs Aged Suture. Statistical analysis: Wilcoxon Rank Sum test via the FindMarkers function in the Seurat R package. Upregulated (pink: $p < 0.05$; red: FDR-adjusted $p < 0.05$), downregulated (cyan: $p < 0.05$; teal: FDR-adjusted $p < 0.05$), and non-significant (ns) genes are displayed on axes of log fold expression (y, avg_log2FC) against p transformed as its negative log value. (**E**) UCell Signature Scoring of transcript expression of genes relating to Complex I electron transport chain (GO-term: 0005747) in the CD90$^+$ clusters of the periosteum and (**F**) suture. Statistical analysis: Uncorrected Dunn's tests; n ≥ 242 cells in periosteum per age group, n ≥ 39 cells in suture per age group. (**G**) UCell Signature Scoring of transcript expression of transcription factors Dbp + Tef + Hlf themselves and (**H**) their combined transcriptional gene targets in the CD90$^+$ clusters of the periosteum. Statistical analysis: Uncorrected Dunn's tests; (**G**) n ≥ 194; (**H**) n ≥ 194. (**I**) UCell Signature Scoring of transcript expression of transcriptional targets of Runx2 in the P1 CD90$^+$ cluster subset of the periosteum. Statistical analysis: Uncorrected Dunn's tests; n ≥ 46 cells per condition. (**J-M**) UMAP-coordinate plot of the CD90$^+$ cells in the periosteum (**J, K**) and suture mesenchyme (**L, M**) for the glycolysis and lipid UCell Signature scoring.

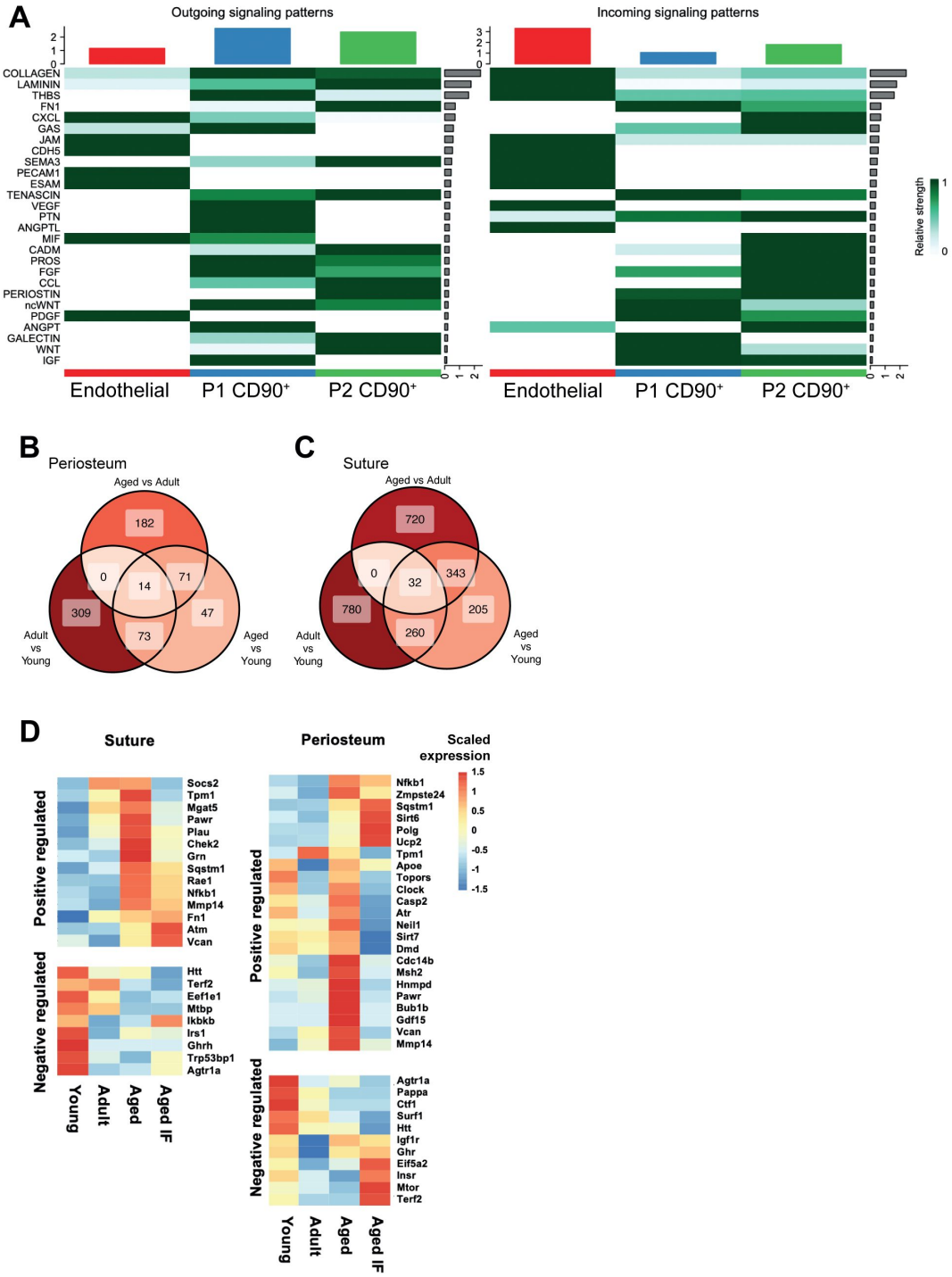


Figure 2 – figure supplement 4.

(**A**) CellChat analysis of the relative inferred levels of outgoing and incoming signaling between subsets of periosteal cells, grouped into pathways. P2 contains the Igf1^{high} and Igf1^{low} populations. Green color scale shows relative intensity in outgoing (left) and incoming (right) inferred signaling patterns for pathways shown at the left edge. Red/blue/green boxes at the top show the relative sum of all pathways. (**B**) Three-way Venn diagrams showing the overlap of statistically-significant positively-upregulated differentially-expressed genes ($p < 0.05$) in comparisons between the CD90$^+$ clusters in the periosteum and (**C**) suture of Young, Adult, and Aged mice. Color of shading and label shows number of genes shared. The central section shows the number of genes conserved throughout all comparisons. (**D**) Heatmaps of gene expression in CD90$^+$ cells from suture and periosteum mesenchyme of young, adult, aged and aged + IF mice. Scaled expression of genes positively regulated and negatively regulated in senescent cells.

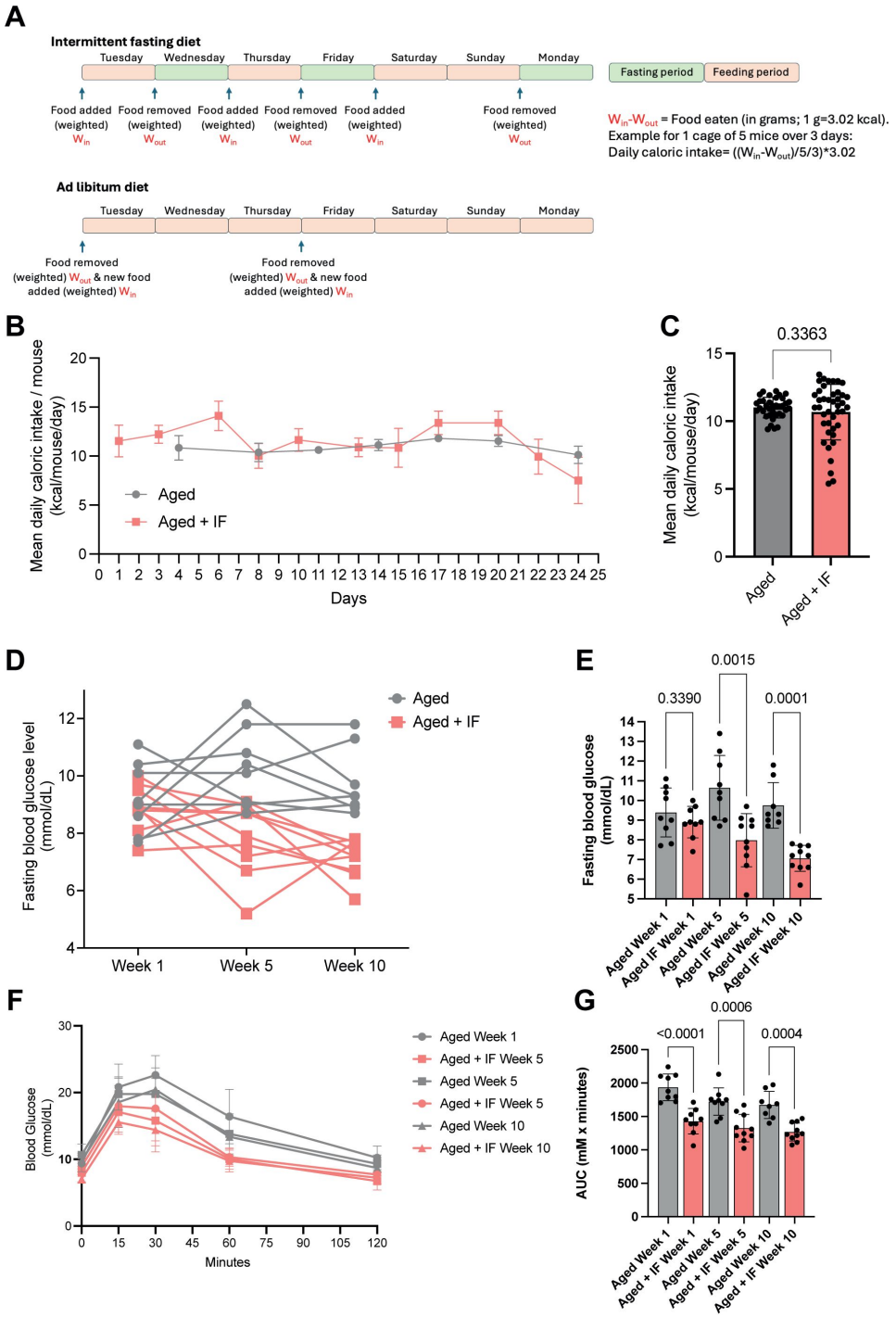
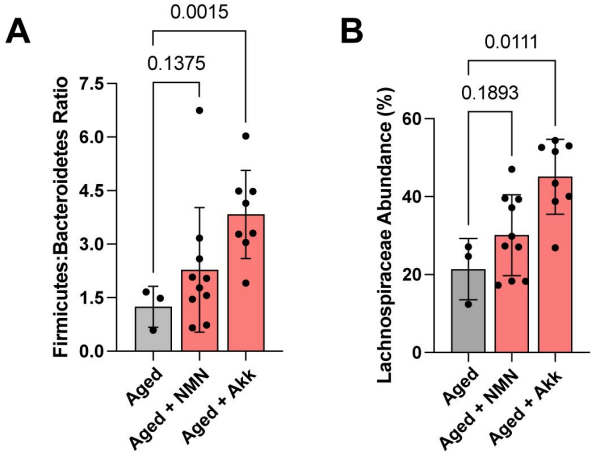


Figure 3 – figure supplement 1.

(**A**) Schematic explaining the approach and measure of the food intake transcripted into caloric intake for the AL and IF diets protocols. (**B**) Calculated daily caloric intake for mouse with ad libitum diet or intermittent fasting diet for the first 24 days of treatment. For each time point, n cage ≥ 3 with 1 to 5 mice/cage. (**C**) Mean daily caloric intake over the 24 days. Data are expressed as mean ± SD with Welch's t-test n ≥ 36. (**D, E**) Fasting blood glucose levels at Weeks 1, 5, and 10 post surgeries. n ≥8 mice/group. (**D**) Connecting curve representation of the fasting blood glucose levels. Each data point is a single dosage; dosages from a same mouse are connected with a line. (**E**) Group comparison for each time point. Data are expressed as mean ± SD with Welch's ANOVA test. (**F, G**) Blood glucose levels during intraperitoneal glucose tolerance test (IPGTT). (**F**) Blood glucose levels over time during the IPGTT. Data are expressed as mean ± SD with n ≥8 mice/group. (**G**) Area under the curves (AUC) of the IPGTT. Data are expressed as mean ± SD with Welch's ANOVA test. n ≥8 mice/group.

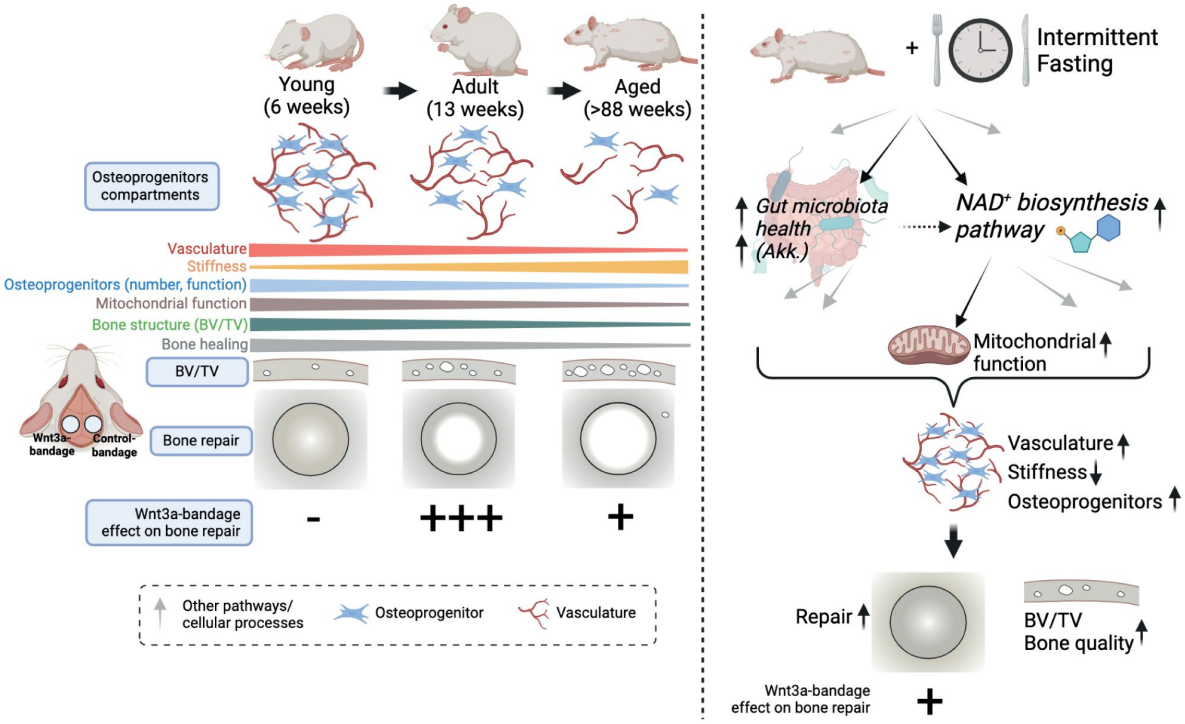
Figure 5 – figure supplement 1

(A) The relative abundance of Firmicutes to Bacterioides bacteria and (**B**) Lachnospiraceae bacteria, as measured by 16S metagenomic profiling. Statistical analysis: Welch's tests on n = 3 (Aged), n = 10 (Aged + NMN), n = 8 (Aged + Akk).



Appendix 2 – figure 1.

Schematic that recapitulates the mains findings of the study. Created in BioRender.Habib, S. (2024) *https://BioRender.com/z14r452*.



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Reviewer #1 (Public review):

Aging reduces tissue regeneration capacity, posing challenges for an aging population. In this study, the authors investigate impaired bone healing in aging, focusing on calvarial bones, and introduce a two-part rejuvenation strategy. Aging depletes osteoprogenitor cells and reduces their function, which hinders bone repair. Simply increasing the number of these cells does not restore their regenerative capacity in aged mice, highlighting intrinsic cellular deficits. The authors' strategy combines Wnt-mediated osteoprogenitor expansion with intermittent fasting, which remarkably restores bone healing. Intermittent fasting enhances osteoprogenitor function by targeting NAD+ pathways and gut microbiota, addressing mitochondrial dysfunction - an essential factor in aging. This approach shows promise for rejuvenating tissue repair, not only in bones but potentially across other tissues.

This study is exciting, impressive, and novel. The data presented is robust and supports the findings well.

https://doi.org/10.7554/eLife.104068.2.sa3

Reviewer #2 (Public review):

Reeves et al explore a model of bone healing in the context of aging. They show that intermittent fasting can improve bone healing, even in aged animals. Their study combines a 'bone bandage' which delivers a canonical Wnt signal with intermittent fasting and shows impacts on the CD90 progenitor cell population and the healing of a critical-sized defect in the calvarium. They also explore potential regulators of this process and identify mitochondrial dysfunction in the age-related decline of stem cells. In this context, by modulating NAD+ pathways or the gut microbiota, they can also enhance healing, hinting at an effect mediated by complex impacts on multiple pathways associated with cellular metabolism.

The study shows a remarkable finding: that age-related decreases in bone healing can be restored by intermittent fasting. There is ample evidence that intermittent fasting can delay aging, but here the authors provide evidence that in an already-aged animal, intermittent fasting can restore healing to levels seen in younger animals. This is an important finding as it may hint at the potential benefits of intermittent fasting in tissue repair.

https://doi.org/10.7554/eLife.104068.2.sa2

Reviewer #3 (Public review):

Summary:

This study aims to address the significant challenge of age-related decline in bone healing by developing a dual therapeutic strategy that rejuvenates osteogenic function in aged calvarial bone tissue. Specifically, the authors investigate the efficacy of combining local Wnt3a-mediated osteoprogenitor stimulation with systemic intermittent fasting (IF) to restore bone repair capacity in aged mice. The highlights are:

(1) Novel Approach with Aged Models:
This pioneering study is among the first to demonstrate the rejuvenation of osteoblasts in significantly aged animals through intermitted fasting, showcasing a new avenue for regenerative therapies.

(2) Rejuvenation Potential in Aged Tissues:
The findings reveal that even aged tissues retain the capacity for rejuvenation, highlighting the potential for targeted interventions to restore youthful cellular function.

(3) Enhanced Vascular Health:
The study also shows that vascular structure and function can be significantly improved in aged tissues, further supporting tissue regeneration and overall health.
Through this innovative approach, the authors seek to overcome intrinsic cellular deficits and environmental changes within aged osteogenic compartments, ultimately achieving bone healing levels comparable to those seen in young mice.

Strengths:

The study is a strong example of translational research, employing robust methodologies across molecular, cellular, and tissue-level analyses. The authors leverage a clinically relevant, immunocompetent mouse model and apply advanced histological, transcriptomic, and functional assays to characterise age-related changes in bone structure and function. Major strengths include the use of single-cell RNA sequencing (scRNA-seq) to profile osteoprogenitor populations within the calvarial periosteum and suture mesenchyme, as well as quantitative assessments of mitochondrial health, vascular density, and osteogenic function. Another important point is the use of very old animals (up to 88 weeks, almost 2 years) modelling the human bone aging that usually starts >65 yo. This comprehensive approach enables the authors to identify critical age-related deficits in osteoprogenitor number, function, and microenvironment, thereby justifying the combined Wnt3a and IF intervention.

[Editors' note: The manuscript was evaluated positively by all three reviewers originally. In the revised manuscript, the authors included some new data following the reviewers' suggestions, while other comments were clarified in the response to the reviewers, and by revising the manuscript text. The new data further support the major conclusions of the paper.]

https://doi.org/10.7554/eLife.104068.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Aging reduces tissue regeneration capacity, posing challenges for an aging population. In this study, the authors investigate impaired bone healing in aging, focusing on calvarial bones, and introduce a two-part rejuvenation strategy. Aging depletes osteoprogenitor cells and reduces their function, which hinders bone repair. Simply increasing the number of these cells does not restore their regenerative capacity in aged mice, highlighting intrinsic cellular deficits. The authors' strategy combines Wnt-mediated osteoprogenitor expansion with intermittent fasting, which remarkably restores bone healing. Intermittent fasting enhances osteoprogenitor function by targeting NAD+ pathways and gut microbiota, addressing mitochondrial dysfunction - an essential factor in aging. This approach shows promise for rejuvenating tissue repair, not only in bones but potentially across other tissues.*

> *Strengths:*
>
> *This study is exciting, impressive, and novel. The data presented is robust and supports the findings well.*
>
> *Weaknesses:*
>
> *As mentioned above the data is robust and supports the findings well. I have minor comments only.*

We thank the reviewer for their enthusiastic and positive assessment of our study. We appreciate the recognition of the novelty and robustness of our data and findings. We have carefully considered the reviewer's comments and have revised the manuscript accordingly. We believe these revisions further strengthen the clarity and impact of our work.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Reeves et al explore a model of bone healing in the context of aging. They show that intermittent fasting can improve bone healing, even in aged animals. Their study combines a 'bone bandage' which delivers a canonical Wnt signal with intermittent fasting and shows impacts on the CD90 progenitor cell population and the healing of a critical-sized defect in the calvarium. They also explore potential regulators of this process and identify mitochondrial dysfunction in the age-related decline of stem cells. In this context, by modulating NAD+ pathways or the gut microbiota, they can also enhance healing, hinting at an effect mediated by complex impacts on multiple pathways associated with cellular metabolism.*
>
> *Strengths:*
>
> *The study shows a remarkable finding: that age-related decreases in bone healing can be restored by intermittent fasting. There is ample evidence that intermittent fasting can delay aging, but here the authors provide evidence that in an already-aged animal, intermittent fasting can restore healing to levels seen in younger animals. This is an important finding as it may hint at the potential benefits of intermittent fasting in tissue repair.*
>
> *Weaknesses:*
>
> *The authors explore potential mechanisms by which the intermittent fasting protocol might impact bone healing. However, they do not identify a magic bullet here that controls this effect. Indeed, the fact that their results with intermittent fasting can be replicated by changing the gut microbiota or modulating fundamental pathways associated with NAD, suggests that there is no single mechanism that drives this effect, but rather an overall complex impact on metabolic processes, which may be very difficult to untangle.*

We thank the reviewer for their positive assessment of our study and for highlighting the significant finding that intermittent fasting can restore age-related declines in bone healing. We appreciate the observation that our results suggest a complex interplay of metabolic processes rather than a single "magic bullet" mechanism. Indeed, the ability of gut microbiota modulation or NAD+ pathway targeting to replicate intermittent fasting's benefits underscores this complexity. While we recognize the challenges of disentangling these interconnected pathways, we believe our findings offer valuable insights into the multifaceted nature of intermittent fasting's impact on aged tissue repair. We hope this study

serves as a foundation for future research aimed at identifying the individual contributions of these pathways and developing targeted therapeutic strategies.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This study aims to address the significant challenge of age-related decline in bone healing by developing a dual therapeutic strategy that rejuvenates osteogenic function in aged calvarial bone tissue. Specifically, the authors investigate the efficacy of combining local Wnt3a-mediated osteoprogenitor stimulation with systemic intermittent fasting (IF) to restore bone repair capacity in aged mice. The highlights are:*
>
> *(1) Novel Approach with Aged Models:*
>
> *This pioneering study is among the first to demonstrate the rejuvenation of osteoblasts in significantly aged animals through intermitted fasting, showcasing a new avenue for regenerative therapies.*
>
> *(2) Rejuvenation Potential in Aged Tissues:*
>
> *The findings reveal that even aged tissues retain the capacity for rejuvenation, highlighting the potential for targeted interventions to restore youthful cellular function.*
>
> *(3) Enhanced Vascular Health:*
>
> *The study also shows that vascular structure and function can be significantly improved in aged tissues, further supporting tissue regeneration and overall health.*
> *Through this innovative approach, the authors seek to overcome intrinsic cellular deficits and environmental changes within aged osteogenic compartments, ultimately achieving bone healing levels comparable to those seen in young mice.*
>
> *Strengths:*
>
> *The study is a strong example of translational research, employing robust methodologies across molecular, cellular, and tissue-level analyses. The authors leverage a clinically relevant, immunocompetent mouse model and apply advanced histological, transcriptomic, and functional assays to characterise age-related changes in bone structure and function. Major strengths include the use of single-cell RNA sequencing (scRNA-seq) to profile osteoprogenitor populations within the calvarial periosteum and suture mesenchyme, as well as quantitative assessments of mitochondrial health, vascular density, and osteogenic function. Another important point is the use of very old animals (up to 88 weeks, almost 2 years) modelling the human bone aging that usually starts >65 yo. This comprehensive approach enables the authors to identify critical age-related deficits in osteoprogenitor number, function, and microenvironment, thereby justifying the combined Wnt3a and IF intervention.*
>
> *Weaknesses:*
>
> *One limitation is the use of female subjects only and the limited exploration of immune cell involvement in bone healing. Given the known role of the immune system in tissue repair, future studies including a deeper examination of immune cell dynamics within aged osteogenic compartments could provide further insights into the mechanisms of action of IF.*

We thank the reviewer for their thorough summary and positive assessment of our study, particularly highlighting its translational nature, the robust methodologies employed, and the

relevance of our aged animal model. We appreciate the insightful suggestion to include male subjects and to explore immune cell dynamics in future investigations.

We acknowledge the limitation of using only female mice in the current study and agree that future studies incorporating both sexes and investigating immune cell contributions within aged osteogenic compartments would offer valuable insights into the mechanisms underlying intermittent fasting and its impact on bone healing.

Our focus on female mice was informed by their distinct characteristics, including delayed healing and higher fracture risk (PMID: 37508423, PMID: 34434120). Importantly, female mice present a more challenging case for bone repair, making them a stringent test for evaluating the effectiveness of our rejuvenation approaches. Moreover, our research protocol, approved under animal license, adhered to ethical principles and the 3Rs, allowing us to reduce the number of animals required by focusing on a single sex.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) The authors should provide a justification for the use of female mice in this study. Additionally, the section on animal methods should be expanded to align with ARRIVE guidelines.*

We thank the reviewer for their valuable feedback. In response to the comment regarding the use of female mice, we have included a justification in the updated manuscript. As noted, female mice were selected for this study due to their distinct characteristics, such as delayed healing and higher fracture risk (PMID: 37508423, PMID: 34434120), which provide a more challenging model for evaluating bone repair strategies. We believe this made our study a stringent test of the efficacy of the rejuvenation approaches being investigated.

Additionally, we have revised the animal methods section to ensure it aligns with the ARRIVE guidelines.

> *(2) Intermittent fasting can influence circadian rhythms in various ways. In the RNA-seq data, do the authors observe any changes related to circadian rhythm pathways?*

The reviewer raises an important point regarding the influence of intermittent fasting (IF) on circadian rhythms. Our RNA-seq data revealed significant alterations in circadian rhythm pathways, particularly within the aged periosteal CD90+ cell population during IF. Specifically, the PAR bZip family transcription factors Dbp, Hlf, and Tef ($q < 0.05$) were significantly upregulated, consistent with their established roles as circadian rhythm regulators (PMID: 16814730, PMID: 31428688).

In suture CD90+ cells from the Aged + IF group, Dbp expression was significantly elevated compared to the Aged AL control group. Moreover, several other circadian-controlled genes, including Sirt1, Kat2b, Csnk1e, Ezh2, Fbxw11, and Ucp2 ($p < 0.05$), were also upregulated (Fig. 4b), suggesting enrichment of Clock/Per2/Arntl transcriptional targets, essential components of the circadian clock.

The observed upregulation of circadian rhythm effectors like Dbp, Hlf, and Tef further suggests a potential role for circadian transcription in CD90+ cell rejuvenation and bone repair in aged mice. While previous studies have primarily focused on the role of circadian rhythms in osteoblasts in vitro (PMID: 34579752, PMID: 30290183), our findings provide compelling evidence for their involvement in bone regeneration in vivo, providing compelling evidence for future investigation into this mechanism.

Chip-SEQ studies have shown D-box sites near promoters in Wnt/β-catenin components (e.g. Lrp6, Lrp5, Wnt8a, Fzd4) in pro-osteogenic transcription factor Zbtb16 (and see Fig 5), and in 11 of the 44 mouse collagen genes (PMID: 31428688). These components are known to regulate osteogenesis, and their proximity to circadian-controlled transcription factors suggests a possible overlap between circadian regulation and Wnt signaling in promoting bone repair. Additionally, circadian rhythmicity, stem cell function, and Wnt signaling are interlinked (PMID: 29277155, PMID: 25414671). Food intake is a powerful regulator of the circadian rhythm in several organs (PMID: 11114885, PMID: 32363197), but little is known about the diet-circadian interaction in bone repair. The possibility that circadian transcription can be harnessed to target Aged stem cell function towards bone repair is a promising prospect.

We have incorporated this information in Figure 2 - figure supplement 3G-H, the results section as well as in the discussion.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The authors refer to 'altered cellular mechanobiology', 'age-related changes in mechanobiology', etc. Here, they are using this terminology to refer to changes in F-actin intensity and nuclear shape. While I agree that these measures are indicators of a cellular response to mechanical cues, calling this 'changes in mechanobiology' doesn't sound quite correct to me. 'Mechanobiology' to me, is a field of study. Perhaps the authors should consider changing their terminology.*

We appreciate the reviewer's insightful comment on the terminology used in our manuscript. We agree that the term "mechanobiology" is a broad field of study and using it in the context of changes in F-actin intensity and nuclear shape may be misleading. We have revised the text to better reflect the specific cellular responses to mechanical cues, such as changes in the cytoskeleton and nuclear morphology, rather than referring to them as "altered mechanobiology." The updated terminology more accurately conveys the observed cellular alterations in response to mechanical forces. We have made these adjustments throughout the manuscript for clarity and precision.

> *(2) Three of the measures the authors use to highlight age-related changes (and rejuvenation) in their animal model are F-actin intensity, nuclear shape, and vascularisation. However, they never really explain what they believe these readouts mean practically/functionally. Indeed, it makes sense that less vascularisation would be associated with an aged phenotype and preclude healing, but this is only mentioned somewhat cursorily in the discussion. While vascularisation is discussed in the context of aging in the discussion, it is not discussed in the context of healing (which would seem relevant in the context of vascularisation being used as a readout in the healing models in response to Akk and IF treatment). Similarly, the changes in F-actin intensity and nuclear shape might suggest changes in the stiffness of the periosteum (as mentioned in the discussion), which could indeed be an indicator of an aged phenotype; however, their role in healing (in response to Akk and IF) are not clearly articulated.*

We appreciate the reviewer's insightful comments and have made revisions to clarify the implications of age-related changes in vascularization, F-actin intensity, and nuclear shape, as well as the functional significance of these observations in the context of healing and rejuvenation.

Vascularization:

Vascularization and modulation of blood flow are critical for calvarial bone repair, as supported by multiple studies (e.g., PMID: 38032405, PMID: 21156316, PMID: 25640220). Early

in the calvarial repair process, blood vessels grow independently of osteoprogenitor cells, establishing a supportive environment that promotes osteoprogenitor migration and subsequent ossification (PMID: 38834586). Furthermore, angiogenic vessels from the periosteum at defect edges contribute to creating a specialized microenvironment essential for bone healing (PMID: 38834586, PMID: 38032405). Compromised vascularization significantly impairs the healing of critical-sized calvarial defects (PMID: 29702250).

Our data reveal a decline in periosteal vascularization with age, potentially compromising this microenvironment and impairing repair in aged animals. Importantly, our findings indicate that intermittent fasting (IF) reverses this phenotype by restoring periosteal vascularization. This rejuvenation of the vascular microenvironment aligns with improved bone repair outcomes in aged mice subjected to IF. We have revised the manuscript to emphasize the importance of vascularization in healing and to highlight the role of IF in restoring this critical aspect of the bone healing microenvironment.

F-actin intensity and nuclear shape:

Age-related changes in F-actin intensity and nuclear shape are associated with increased tissue stiffness, a hallmark of aging. Tissue stiffness has been shown to impair progenitor cell function and hinder repair in various systems, including neuroprogenitors (PMID: 31413369). Softening the extra cellular matrix in aged tissues has been demonstrated to partially restore progenitor function and improve repair outcomes, as seen in the case of neuroprogenitors (PMID: 31413369). In our study, IF reversed age-associated changes in F-actin expression and nuclear shape, restoring these parameters to a phenotype resembling that of younger animals. This suggests that IF mitigates the mechanical changes associated with aging, reducing tissue stiffness and rejuvenating the periosteum to facilitate improved bone healing, similar to the outcomes observed in younger models.

Following the reviewer's advice, we have revised the text to clearly articulate the correlations and interpretations of our data regarding tissue mechanics and bone repair. Thank you for highlighting these critical aspects.

> *(3) In relation to my point 2) on nuclear shape, there are reports that aging is linked to changes in Lamin B1. Have the authors considered this? It might provide a clearer link between their data and the tissue-level phenotypes they observe.*

Thank you for your comment regarding the potential link between aging and changes in Lamin B1. Following your suggestion, we performed Lamin B1 immunostaining on samples from Young, Adult, Aged, and Aged + IF groups. However, no significant differences in Lamin B1 levels were observed across these groups. These findings indicate that changes in Lamin B1 in osteoprogenitors are not apparent during aging, suggesting that Lamin B1 alterations in the context of aging may be tissue- and cell-type-specific.

The new data was added in Figure 1 - figure supplement 2i-j.

> *(4) In the data associated with Figure 2, the authors find that in the aged mice, MMP9 expression is increased, but MMP2 expression is decreased. They associate the decrease in MMP2 expression with decreased migration, but the canonical function of MMP9 should be similar to that of MMP2. Are there tissue-specific differences in the activity of MMP2/9 that could account for this?*

Thank you for the thoughtful comment. While both MMP-2 and MMP-9 are involved in ECM remodeling and share some overlapping canonical functions, their roles are context-dependent and exhibit tissue-specific differences that could explain the observed changes in aged mice. MMP-2 has been shown to play a critical role in maintaining the structural and functional integrity of flat bones, such as those in the craniofacial skeleton, by supporting

bone remodeling (PMID: 17400654, PMID: 17440987, PMID: 16959767). The decreased expression of MMP-2 in aged mice may impair these local processes, leading to reduced migratory capacity of osteoprogenitors and contributing to aging-related changes in flat bone structure and function.

In contrast, MMP-9 is more prominently involved in long bone remodeling, particularly at the growth plate where it regulates hypertrophic chondrocyte turnover, vascularization, and ossification during endochondral bone formation (PMID: 21611966, PMID: 9590175, PMID: 23782745, PMID: 16169742). Additionally, MMP-2 and MMP-9 differ in their regulation of specific ECM substrates and their interactions with bone-resident cells, which may further drive divergent outcomes in distinct bone types. For example, MMP-9's role in osteoclastogenesis and its regulation of ECM proteins like type I collagen could be more critical in long bones, while MMP-2's involvement in fine-tuning ECM microarchitecture may hold greater importance in flat bones.

The increased expression of MMP-9 in aged calvarial osteoprogenitors may reflect a compensatory mechanism in response to the reduced MMP-2 activity, possibly in response to increased ECM turnover demands. Further studies examining the precise molecular pathways driving these changes in osteoprogenitors will help clarify the underlying mechanisms and their contributions to age-related alterations in flat bone structure and function.

> *(5) In lines 391-2, the authors conclude that the data from Figure 4 shows that "during IF, CD90 cells, despite being aged, are more capable of ECM modulation and migration". The authors certainly present evidence that this is true, but the RNAseq showed that the enriched GO terms were predominantly associated with immune responses ('response to cytokine') and the proliferation phenotype seems very strong. Therefore, I would suggest that this overarching statement regarding the findings be less focussed on this one aspect of the finding, which doesn't look to be the dominant phenotype of the cellular response. And indeed, the authors move on from here to explore a mechanism associated with metabolism, not specifically with ECM remodelling.*

We greatly appreciate the reviewer insight regarding the interpretation of our findings, particularly the conclusion drawn from Figure 4.

In response, we have revised the conclusion to more accurately reflect these findings.

The revised text in the conclusion now reads: " Together, these findings suggest that IF rejuvenates aged CD90+ cells, in part, by enhancing proliferation, immune response, ECM remodeling, Wnt/β-catenin pathway, and metabolism, including increased ATP levels and decreased AMPK levels."

We hope that this adjustment better aligns with your suggestion and provides a more accurate summary of the key findings.

> *(6) Fasting blood glucose levels are often cited as an indicator of metabolic health. Did the authors look at this in their animals who underwent the IF protocol? Could this have had an impact on the healing response?*

We thank the reviewer for this insightful comment. Throughout our study, we have withdrawn blood from the animals for various analyses that were not included in this manuscript in order to maintain focus on the osteoprogenitors.

Our analysis included the assessment of the metabolic health of the animals using fasting blood glucose levels and the area under the curve (AUC) of the intraperitoneal glucose tolerance test (IPGTT).

Fasting blood glucose levels reflect the animals' ability to maintain stable glucose levels after fasting, while the AUC from the IPGTT measures how efficiently glucose is cleared from the bloodstream following a glucose challenge. Typically, lower fasting blood glucose levels and reduced AUC indicate improved insulin sensitivity, better glucose metabolism, and enhanced metabolic control (PMID: 18812462, PMID: 19638507).

Our findings show that intermittent fasting (IF) significantly reduced both the fasting blood glucose levels and the AUC in the IPGTT. This indicates that IF enhances metabolic flexibility, likely through improved insulin sensitivity and better glucose homeostasis. By lowering fasting blood glucose, IF reduces the reliance on excessive gluconeogenesis during fasting, while a reduced AUC indicates more efficient postprandial glucose clearance, consistent with enhanced insulin action and reduced fluctuations in blood glucose levels. The new data has been incorporated in Figure 3 - figure supplement 1d-g.

Methods:

“Blood glucose level measurement

Fasting blood glucose levels were measured (Accu-Check tests strips) from 6h fasting mice by blood sampling the tail vein. For intraperitoneal glucose tolerance test (IPGTT), glucose was injected intraperitoneally (2 g/kg), and the blood glucose levels were measured after 15, 30, 60 and 120 minutes.”

Improved metabolic health through lower fasting glucose and reduced AUC can have profound implications for tissue repair (PMID: 32809434). Stable glucose levels ensure a consistent energy supply for key cellular processes, such as cell proliferation, migration, and differentiation, which are essential for regeneration. Enhanced insulin sensitivity supports nutrient delivery to cells and reduces inflammation, creating an environment conducive to tissue healing. Additionally, intermittent fasting's ability to optimize glucose metabolism and regulate insulin secretion may enhance the function of stem and progenitor cells, further improving the tissue repair process (PMID: 28843700). Together, these findings suggest a mechanistic link between improved metabolic health and the enhanced healing observed in animals subjected to intermittent fasting.

> *(7) In Supplementary Figure 10, the authors look at bone remodelling by assessing TRAP staining, as an indicator of osteoclast activity. I'm not sure if these data add all that much to the study. The authors have looked at bone formation at a tissue level using microCT. Here, they look at bone resorption at a cellular level with the TRAP assay. Overall, this probably suggests more bone remodelling, but the TRAP assay on its own at the cellular level could also be interpreted as an osteoporosis-like phenotype. This is clearly not the case because the authors show robust bone healing by microCT. In short, as an isolated measure of osteoclast activity at the cellular level without cellular-level assays of osteoblast activity, the interpretation of these data is not that clear. The microCT speaks far more of the phenotype and is, in my opinion, sufficient to make this point.*

We thank the reviewer for their comments regarding the interpretation of the TRAP staining data and its context within the study. We appreciate the concern that, without direct assays of osteoblast activity, the TRAP assay could lead to ambiguity.

We have shown that intermittent fasting significantly increases the number and function of osteoprogenitor cells, the precursors to osteoblasts. While we acknowledge that these data do not directly measure osteoblast numbers or activity, they strongly suggest an increased capacity for osteoblast differentiation and bone formation. This aligns with the microCT findings of robust bone structure and healing.

After careful consideration and given that the microCT and histology findings already provide robust and comprehensive evidence for bone structure and healing, we have decided to remove the TRAP staining data from the manuscript. We believe this change simplifies the manuscript and strengthens its focus on the most impactful data.

> *(8) In the discussion, the authors make a number of links between aging and IF. However, one of the exciting conclusions of this manuscript is that IF aids in healing in aged animals. In this context, IF has not impacted the aging process itself because the animals have not experienced an IF protocol across their lifespan, but rather only after injury. In this context, perhaps the authors should also be focussing their discussion on evidence of the short-term response to IF rather than its effects on aging, which are longer-term.*

We appreciate the reviewer's comment and agree that emphasizing the short-term effects of intermittent fasting is crucial. Our study is the first to examine this protocol in Aged animals.

To address this, we have revised the discussion and highlighted how short-term IF enhances metabolic health, promotes osteoprogenitor functionality, and supports bone remodeling, as observed in our study.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The authors should clarify details on intermittent fasting protocols, especially regarding caloric intake differences between fasting and non-fasting days, to aid reproducibility.*

We appreciate the reviewer's suggestions and have incorporated them by clarifying the relevant details. The new data are presented in Figure 3 - figure supplement 1a-c.

Methods:

“Caloric intake calculation

To assess the caloric intake of mice, the food was weighted when made available to the mice (Win), and when removed (Wout). The daily consumed food was calculated based on the weight difference (Win - Wout), then converted to kcal (1 g = 3.02 kcal, LabDiet, 5053), and expressed as kcal/mouse/day for each cage (n cage ³ 3 with 1 to 5 mice/cage).”

> *(2) Did the authors evaluate the effect of their intermittent fasting protocol on fasting blood glucose levels?*

Following the reviewer comment we included two measurements: 1) Fasting blood glucose, which reflects the ability to maintain glucose homeostasis during fasting, and 2) fasting blood glucose levels and the area under the curve (AUC) of the intraperitoneal glucose tolerance test (IPGTT), which measures glucose clearance efficiency after a glucose challenge. Lower values for both typically indicate improved insulin sensitivity, glucose metabolism, and metabolic control.

Our findings demonstrate that intermittent fasting significantly reduced both fasting blood glucose and IPGTT AUC, suggesting enhanced metabolic flexibility, likely through improved insulin sensitivity and glucose homeostasis. Lower fasting blood glucose with IF indicates reduced reliance on gluconeogenesis during fasting, while a reduced AUC suggests more efficient postprandial glucose clearance, consistent with enhanced insulin action and reduced blood glucose fluctuations. This new data is included in Figure 3 - figure supplement 1.

Generally, the improved metabolic environment supports tissue repair by ensuring adequate energy for cell proliferation and migration, reducing inflammation, and promoting the

function of stem cells involved in tissue regeneration. Thus, this outcome of intermittent fasting may create a more favorable environment for tissue repair, potentially accelerating the healing of damaged tissues and improving overall regenerative capacity.

> *(3) In Figure 1E-F, the nuclei have an interesting shape and the authors quantified F-actin. Given the role of lamin B in nuclear integrity, an analysis of lamin B expression and its structural integrity in aged osteoprogenitors could provide valuable insights into cellular aging mechanisms and their potential reversal with intermittent fasting.*

In response to the reviewer's comment, we performed Lamin B1 immunostaining on samples from Young, Adult, Aged, and Aged + IF groups. We observed no significant differences in Lamin B1 levels across these groups. This suggests that age-related changes in Lamin B1 are not evident in osteoprogenitors and may be tissue- or cell-type specific. The new data was added in Figure 1 - figure supplement 2i-j.

> *(4) The authors should explain, in the main text or the methods section, why are they only using females in this study.*

We appreciate the reviewer's comment regarding the use of female mice. Female mice were chosen for this study due to their delayed healing and higher fracture risk (PMID: 37508423, PMID: 34434120), presenting a more challenging model for evaluating bone repair strategies and providing a stringent test of our rejuvenation approaches. This justification has been added to the revised manuscript. The animal methods section has also been updated to comply with ARRIVE guidelines.

> *(5) This story stands alone and has an incredible amount of data. However, for a follow-up study, I would like to suggest consideration of including a broader analysis of immune cell involvement within the osteogenic compartments to strengthen the mechanistic understanding of IF's impact.*

We thank the reviewer for this insightful suggestion. We agree that investigating the role of immune cells within the osteogenic compartments could provide valuable mechanistic insights into how intermittent fasting influences tissue regeneration. Immune cells are key mediators of inflammation and repair, and their interactions with osteoprogenitors and other cells in the bone healing environment likely contribute to IF's effects.

While our study focuses on IF's impact on osteoprogenitor function and tissue repair, we acknowledge the importance of future research exploring immune cell involvement. Techniques like single-cell RNA sequencing or flow cytometry could characterize immune cell populations and their functional states within osteogenic niches, allowing for a deeper understanding of immune-skeletal interactions during IF-mediated bone healing. We appreciate the reviewer highlighting this promising avenue for future research.

> *Minor corrections to the text and figures:*
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> *(1) References formatting should be revised (eg. line 41).*

The reference formatting was corrected.

> *(2) Line 144 - what do the authors mean by p2 in the references?*

Thank you for your comment, we corrected the error and removed p2 from the reference.

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