

Analysis of Age and Sex-Specific Differences in Gene and pri-miRNA Expression in the Cell-Types of the *Mus Musculus* Subventricular Zone

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Biology, Cell Biology, and Biochemistry at Brown University

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This dissertation by Amy Elizabeth Elias is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Chapter 1:
Introduction

Aging is the costly and detrimental process of progressive deterioration and loss of biological homeostasis with time, resulting in death. There are conserved hallmarks of the biological aging process that give rise to age-associated dysfunction: altered intercellular communication, stem cell exhaustion, cellular senescence, mitochondrial dysfunction, deregulated nutrient sensing, loss of proteostasis, epigenetic alterations, telomere attrition, and genomic instability (1). With age, animals are at greater risk of developing and succumbing to neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and Amyotrophic Lateral Sclerosis among many others (2). There is a great interplay between all the hallmarks which ultimately results in systemic aging and the onset of these neurodegenerative disease and age-associated dysfunction. During neurodegenerative disease, neurons are lost, demyelination is common, and there is a decline in neural stem cell activity and brain volume (3-7). During the normal aging process, there is also a natural decline in cognition, decline in neural stem cell activity, and decline in brain volume (8-12). Since normal aging and age-associated neurodegenerative disease share many of the same characteristics, understanding the source of this neural stem cell decline and inability to produce new neurons is imperative. With a specific focus on neural stem cell (**NSC**) aging, it is paramount to focus on how changes in gene expression lead to alterations in several hallmarks resulting in their exhaustion and decline. Likewise, the role of regulatory RNAs on gene-expression and changes within each cell-type regulating the NSCs is underscored by the lack of data available in aging.

Neurogenic Niches and Neural Stem Cell Differentiation in the Mouse

Neural stem cells (**NSCs**), which self-renew and give rise to new neural cell-types, are found within the subventricular zone (**SVZ**) of the lateral ventricles and the subgranular zone (**SGZ**) of the hippocampus dentate gyrus (**DG**) (13-18). Although both regions are sites of neurogenesis, the NSCs of the SVZ are multipotent, giving rise to neurons, astrocytes, or oligodendrocytes, while the neuronal precursor cells of the DG are unipotent (13, 19-24).

Additionally, the SVZ is the larger of the two neurogenic niches in the adult brain. Neurogenesis persists past development stages and continues into adulthood through the lifelong process of adult neurogenesis (14, 15, 25-28). In the rodent SVZ, NSCs are known as type B cells, and they are a stem cell subset of glial cells, maintaining expression of glial fibrillary acidic protein (**GFAP**), and capable of giving rise to progenitors (13, 21, 29). These cells interact with several other niche cell-types, blood vessels, and the wall of the lateral ventricle making contact with the cerebrospinal fluid (**CSF**) in rodents and humans (30, 31).

Type B cells lacking expression of Nestin are in a quiescent state while type B cells expressing Nestin exist in an activated state and are capable of asymmetric division to self-renew and give rise to type C cells known as transit amplifying cells (**TAPs**) (32, 33). TAPs are distinguished by their expression of *Ascl1* and *Dlx2* and upregulation of EGFR (30, 32, 34). Furthermore, growth factors such as EGF influence proliferative abilities and lineages of TAPs (17, 35). For example, NSCs in the presence of EGF express oligodendrocyte progenitor cell (OPC) markers, while the removal of EGF leads to more neuronal expression patterns in the TAPs (35). Under normal circumstances, oligodendrogenesis is used to supply the corpus callosum with new oligodendrocytes (20). This process allows for the production of oligodendrocyte precursor cells (**OPCs**), which give rise to committed oligodendrocyte progenitors (**COPs**) and then myelin forming oligodendrocytes (**MFOLs**) (36). Following demyelination injury, however, oligodendrogenesis can replenish oligodendrocytes near the lesion, and after stroke injury, astrogliogenesis is favored in the SVZ and astrocytes can migrate to lesion sites (37, 38).

When favoring neurogenesis, however, TAPs divide 3-4 times before differentiating into neuroblasts known as type A cells (39). Neuroblasts migrate in chains along the rostral migratory stream to the olfactory bulb, guided by cell autonomous Robo/Slit signaling and cell non-autonomous signaling with astrocytes, cells within the olfactory bulb, and cells within the SVZ to repel neuroblasts from the SVZ and attract them towards to the olfactory bulb (40-42).

Once in the olfactory bulb, neuroblasts differentiate into interneurons, which requires specific cues from surrounding cells (42). If neuroblasts are transplanted elsewhere in the brain, they do not differentiate into neurons but rather glia (43, 44). During this highly regulated process, extrinsic signaling and cell-cell communication is indispensable for proper neurogenesis, astrogliogenesis, and oligodendrogenesis from the SVZ.

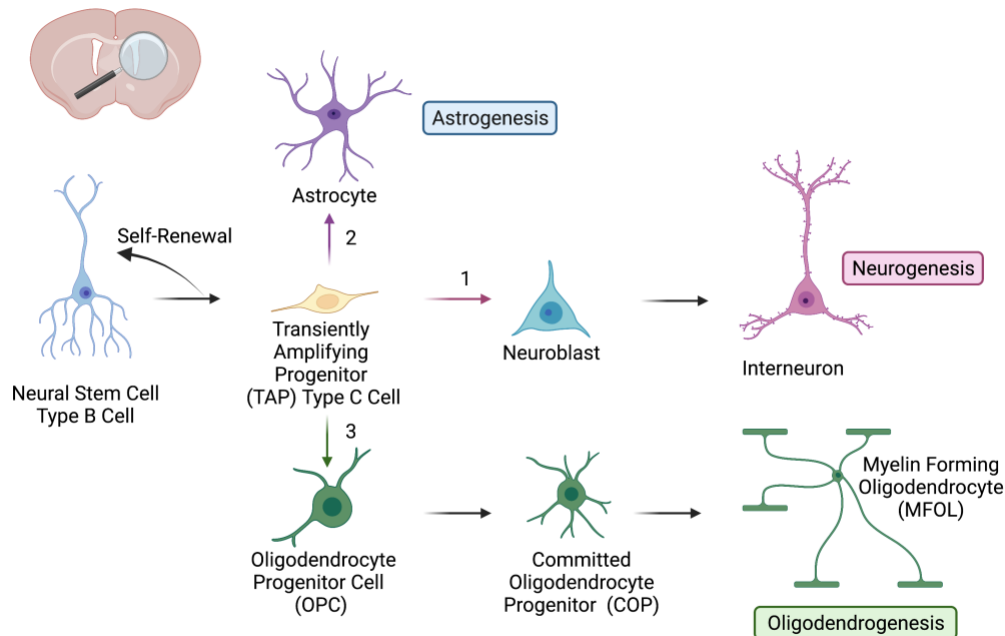


Figure 1: NSC Proliferation and Differentiation in the SVZ. NSCs can divide asymmetrically to self-renew and give rise to a TAP. In neurogenesis (1), the TAP gives rise to neuroblasts which mature into interneurons. In astrogenesis (2), TAPs give rise to astrocytes. In oligodendrogenesis (3), TAPs give rise to OPCs which become committed as COPs and give rise to oligodendrocytes.

SVZ Composition and Cytoarchitecture in the Mouse and Human

Along with the neural stem cells and their progenitors, there are ependymal cells, non-neurogenic astrocytes, oligodendrocytes, vasculature, pericytes, microglia/macrophages within

the subventricular zone (30, 45, 46). In the mouse SVZ, ependymal cells line the ventricle wall and are in contact with the CSF. These ependymal cells act as a barrier for the niche, although NSCs do contact the CSF itself (31). Moreover, ependymal cells additionally play a paramount signaling role by secreting pigment epithelium derived factor (PEDF) to support self-renewal of NSCs and Noggin to foster a neurogenic environment for the NSCs (47, 48). Additionally, within the SVZ, there are non-neurogenic astrocytes (30, 49). The close contact of NSCs and astrocytes is necessary for neurogenesis, as shown through co-culture or conditioned medium experiments (50, 51). Furthermore, astrocytes from this region are specialized and have specific signal secretion to allow for a neurogenic environment since transplantation experiments have shown that neurogenesis relies on signals from within the niche (44, 52). Along with contacts with surrounding astrocytes and the CSF, NSCs make contact with blood vessels in the SVZ (30, 49, 53). The blood vessels in the SVZ have areas covered in astrocytes and pericytes, cells which regulate blood-brain-barrier (BBB) interactions, and they have additional areas absent of both cell-types that are populated with NSCs (54). The areas of blood vessel contact with NSCs tend to have greater secretion of molecules into the SVZ, and are termed “leaky”, thereby showing the importance of this interaction (54, 55). Furthermore, within the niche there are cells with immune function such as microglia and macrophages. Microglia, the resident “immune” cells of the brain, secrete signals to foster pro-neurogenic environment at baseline or reduce neurogenesis during microglial activation as a result of inflammation, demonstrated through co-culture, conditioned medium, and *in vivo* experiments (56, 57).

Although the human and rodent SVZ maintain the same cell-types, their cytoarchitecture has slight yet critical differences. In the human SVZ, there are four distinct layers, the first being the ependymal cell layer interfacing with the CSF of the lateral ventricles. Between the ependymal cells and the neural stem cells and astrocytes, there is a hypocellular gap – devoid of cells, yet containing the processes extending towards the ependymal layer. The third layer

contains the dividing NSCs while layer four contains the barrier dividing astrocytic tissue of the SVZ from tissue belonging to other neural areas (49). In the rodent brain, there is no such gap between ependyma and surrounding cells, and there are no distinct layers (30, 49). In addition to these key properties, the human SVZ has fewer neurons and lacks chain migration of neuroblasts, which is a signature of the rodent SVZ (40, 49, 58). Comparisons of proliferation rates between human and rodent SVZ NSCs have additionally shown that rodents have a higher rate of proliferation than humans (49, 58).

Pathways and Cell-Signaling Regulating Neurogenesis

The potential for NSCs to become activated and lay a trajectory towards neurogenesis, oligodendrogenesis, or gliagenesis is dependent upon paracrine signaling from nearby cells or cells in close contact and alterations to several pathways within the NCSs themselves. Wnt signaling, Notch signaling, BMP signaling, and FoxO signaling are among the most important pathways regulating NSC lineage, proliferation, and self-renewal. In addition, paracrine signaling of growth factors further guide NSC fate decisions.

Wnt Signaling

Wnt signaling is one of the major pathways regulating NSC proliferation and progenitor lineage fate (46, 59-62). Wnt signaling can occur in a canonical or noncanonical fashion. In canonical Wnt signaling, Wnt3a and Wnt7a are secreted and bind to the Frizzled (Fzd) receptor at the cell membrane. Wnt3a is secreted from astrocytes in the SGZ niche while Wnt7a has been shown to be secreted by microglia and astrocytes (62-64). The signal is transduced to Disheveled (Dvl) and, through interaction with co-receptors low-density-lipoprotein-related proteins 5/6 (LRP5/6), transmits the signal that inhibits phosphorylation of β -catenin by GSK3 β . β -catenin accumulates, and the protein is then able to translocate to the nucleus where it binds to transcription factors and leads to increased transcription of target genes (61). When Dickkopf-

1 (Dkk1), an inhibitor of Fzd, is expressed within the SVZ of rodents, there is a sharp decline in the number of neuroblasts formed. When GSK3 β is inhibited pharmacologically, on the other hand, NSC proliferation increased (65). In cases of demyelination injury, however, Wnt7a from microglia transduces a signal leading to NSC oligodendrogenesis to help repair the injury (64).

In the noncanonical pathway, known as the Planar Cell Polarity pathway, Wnt5a binds to Fzd and relays the signal to Dvl without LRP5/6. Dvl interacts with disheveled-associated activator of morphogenesis-1 (Daam1) and activates Rac thus leading to c-Jun N-terminal Kinase (JNK) activation. Furthermore, Daam1 can activate Rho then activating Rho kinase (ROCK) (66). The ultimate result of these signal transduction cascades helps to mature neuroblasts into new neurons in the olfactory bulb (61, 67, 68). Furthermore, non-canonical Wnt signaling helps to maintain the NSC pool via induction of a quiescent state (60).

Notch Signaling

Notch signaling relies on direct cell-cell contact (69, 70). Notch receptors are activated via binding of Delta and Jagged ligands on an adjacent cell. The Notch receptors are transmembrane proteins; thus, when Notch receptor is bound by its ligands, the transmembrane domain is cleaved, and the notch intracellular domain (NICD) is released into the cell. The NICD is then imported to the nucleus, and it binds RBPj, resulting in transcription of bHLH transcription factors, importantly HES family genes (71, 72). Notch helps to maintain the neural stem cell pool and prevents differentiation as shown by studies where RBPj null mice lacked a stem cell pool and ablation of Notch signaling cascade led to an increase in glia (73, 74).

BMP Signaling

Bone Morphogenetic Proteins (BMPs) signal to NSCs to stimulate glial fate specification over neuronal (48). These proteins are members of the TGF- β family, and they transduce signals through BMP receptors leading to phosphorylation of SMAD1/5/8 via BMP type I receptor

kinases. Once phosphorylated, these three Smads then form a complex with Smad4. Thereafter, nuclear translocation takes place, and transcription of target genes takes place. Under normal conditions, BMPs BMP2 and BMP4 promote differentiation of NSCs towards a glial fate (48, 75). Overexpression of BMP7, however, leads to an overall decrease in proliferation(48). The antagonist of BMP, Noggin, is produced by ependymal cells and promotes neurogenesis under normal conditions (48). When BMP4 is inhibited by Noggin under demyelinating conditions, however, phosphorylation of SMAD1/5/8 was markedly decreased leading to an oligodendrocyte fate specification (76). Noggin has a lesser effect on BMP7 expression, on the other hand(48). There is additionally BMP signaling that leads to induction of PI3K and ERK pathways in a Smad-independent manner (77).

FoxO Signaling

The FoxO subset of the Forkhead family contains transcription factors FoxO1, FoxO3, FoxO4, and FOXO6. FoxO1 is expressed in the NSCs of the SVZ and FoxO3 is expressed in neurons, astrocytes, oligodendrocytes, and NSCs of the SVZ (78). In the presence of growth factors or insulin, receptors for these growth factors or insulin will induce a cascade leading to phosphoinositide kinase (PI3K) activation and downstream activation of the kinases belonging to the Akt family (79-81). Akt phosphorylates FoxOs thus causing a translocation from the nucleus to the cytoplasm (80-84). FOXO6, however, remains localized to the nucleus in the presence of growth factors (85). The translocation of other FOXO isoforms is accomplished by 14-3-3 proteins binding to FoxOs, which may help to destabilize their binding to DNA and expose a nuclear export signal (NES) (83, 86). When in the cytoplasm, phosphorylated FoxOs are unable to re-enter the nucleus due to masking of the nuclear localization signal (NLS), and they are additionally degraded through ubiquitination (87-90). In the absence of growth factors, however, FoxOs remain in the nucleus and function as transcription factors (80, 81).

FOXOs play a tremendously important role in preserving the NSC pool (91-94). FoxO1 overexpression in NSCs and neural progenitor cells leads to a decline in glial and neuronal differentiation compared to normal conditions, while a knock-out of FoxO1 leads to an increase in neural differentiation (94). Meanwhile, knock-out of FoxO3 in mice leads to a depletion of NSCs, and the existing NSCs have a limited self-renewal capacity (91). Interestingly, FoxO3 shares many enhancer binding sites with Ascl1, a transcription factor expressed in activated NSCs and neural progenitors (28, 92). FoxO3 prevents Ascl1-dependent transcription of Dll1 and Hes6 in culture while restricting neurogenesis *in vivo* (92). Furthermore, FoxO3 acts as a transcription factor for genes involved in autophagy, within neural progenitor cells, and helps to enhance their autophagic flux as demonstrated via overexpression and knock-out of FoxO3 and use of a LC3-based reporter system to measure autophagic flux (95).

Growth Factors

Epidermal Growth Factor (EGF), Fibroblast growth factor-2 (FGF2), Insulin-like-growth-factor-1 (IGF1), and Vascular endothelial growth factor (VEGF) are among the growth factors involved in neurogenesis in the SVZ. The binding of these secreted growth factors all trigger downstream cascades such as PI3K/Akt and MEK/ERK pathways in the target cell (71). EGF and its' receptor EGFR modulates cell fate of NSCs by promoting their lineage towards TAPs via inhibition of Notch (96). FGF2, secreted from GFAP-positive cells, helps to increase cell-cycle length of a distinct pool of NSCs and knock-out of FGF2 results in decreased olfactory bulb size as well as a reduction in neuroblasts (97, 98). Meanwhile, knock-out of IGF-1 in mice led to detrimental patterning defects in the olfactory bulb and a failure of neuroblasts to migrate from the SVZ. Consequently, IGF-1 helps in the patterning of new neurons in the OB and migration from the SVZ (99). Meanwhile, VEGFR receptors have been found on NSCs and the addition of VEGF to the SVZ increases neurogenesis (100, 101).

Calcium Signaling

Calcium signaling is paramount in cell-cell interaction and intracellular activity in the brain. For instance, store operated calcium entry channels (SOCs), which control calcium flux from the endoplasmic reticulum to the plasma membrane, have been shown to tightly control NSC self-renewal and proliferation activities. When these channels are pharmacologically blocked, asymmetric division increases and the NSC pool is diminished (102). By comparing gene-expression in neuroblasts from different ends of the rostral migratory stream, it was found that calmodulin (Calm1), calmodulin dependent protein kinase 4 (Camk4), and histone deacetylase-2 (Hdac2) were differentially expressed, among others. When knocked down using adenovirus vectors, knockdown's limited neuroblast migration, especially Hdac2 (103). This study underscores the calmodulin network's powerful impact on neuroblast migration. Furthermore, calcium can activate RAS signaling leading to activation of MAPK signaling through calmodulin or tyrosine kinase PYK2 activation. This leads to increased proliferation and differentiation (104). Wnt5a can additionally activate the Wnt/Ca²⁺ pathway leading to increased neural progenitor cell differentiation and survival (105, 106).

miRNA Biogenesis

Pathways regulating NSC self-renewal and differentiation are subject to regulation by small RNAs, which can translationally repress mRNAs, or cause their degradation, and decrease protein abundance of genes within a pathway. Some of the most potent regulators of gene expression are microRNAs (**miRNAs**). miRNAs are generated as primary miRNA transcripts (**pri-miRNAs**) by RNA Polymerase II (107). pri-miRNAs are transcribed as either discrete or polycistronic transcripts (108, 109). Most primary miRNA transcripts are several kb long, much longer than their mature form at 22-25 nucleotides (108, 110, 111). pri-miRNAs exist in a stem loop structure consisting of basal single-stranded segments in the 5' and 3' ends followed by dsRNA segments - forming the lower stem -, upper stem - containing the mature

miRNA sequence -, and terminal loop (112). Following transcription of the pri-miRNA, the microprocessor complex, consisting of RNase III Enzymes Drosha and DGCR8, recognizes the basal segments about 11 bp from the stem and acts as an anchor for Drosha binding to the stem (112, 113). Following the binding of DROSHA and DGCR8, cleavage of the stem loop structure about 11 base pairs from the stem loop structure occurs thus freeing the pre-microRNA (**pre-miRNA**) product from the basal segments (112). Furthermore, cleavage of pri-miRNA paralogs has shown that there are noncanonical sites of cleavage due to flexibility of certain stem loop structures thus leading to downstream differences in the seed sequences of mature miRNAs (114). During this processing stage from primary to pre-miRNA, there are several proteins that can act to promote or inhibit cleavage. For example, the NF90/NF45 complex (ILF3/ILF2) acts to hinder binding of the microprocessor complex thus limiting production of some pre-microRNAs (115). Furthermore, other proteins can enhance primary to pre-miRNA processing by increasing DROSHA activity, like p53, or by interaction with the loop structure to allow for easier cleavage of pri-miRNAs like hnRNPA1 and KRSP (108, 115-118). For miRNAs that exist as introns within a gene (**mitrons**), cleavage by Drosha is unnecessary since splicing machinery will free these pre-miRNA sequence into a lariat, which is then cleaved into the canonical pre-miRNA structure by lariat debranching enzyme (**Ldbr**), giving it a 5' phosphate and 3' hydroxyl group (109, 119).

The resulting pre-miRNA is about 65-70 nucleotides long, and it is exported to the cytoplasm via Exportin5/RanGTP (**XPO5**) (115, 120, 121). XPO5 binds to the stem structure of pre-miRNAs in a non-sequence specific manner and protects the 3' overhang within the two alpha helices joined by a loop known as its' tunnel structure (122). With abundant expression of XPO5, there is an increase in the efficiency of pre-miRNA delivery to the cytoplasm (123). Therefore, between primary microRNA transcription, and pre-microRNA export, there are several intricate levels of regulation that occur within the nucleus.

Within the cytoplasm, Dicer binds to the pre-miRNA via recognition of dsRNA through PAZ domains and cleaves the pre-miRNA through RNase III domains into a 22-25 nucleotide structure (124). The 22-25 nucleotide dsRNA product is the result of the distance between RNase III and PAZ domains within Dicer (124). Dicer is additionally bound to Ago2, HSP90, and TRBP, and GW182 to form the microRNA RISC Loading Complex (miRLC) in humans (125, 126). TRBP helps to determine the cleavage site of the pre-miRNA (125), and HSP90 acts to extend the life of the RISC complex (127). Meanwhile, GW182 proteins are scaffolds for the binding of proteins mediating target mRNA silencing (126). The transient dsRNA duplex is loaded onto Ago2 (125). Once Dicer departs, the complex is called the RNA Induced Silencing Complex (**RISC**). After unwinding of the complex, a dominant strand is selected and the RISC is guided to the 3' UTR of a mRNA complementary the miRNA seed sequence to aid in the silencing of the mRNA (110, 115, 128). The seed sequence is the sequence from nucleotides 2-7 from the 5' end of the miRNA that binds complimentary to the 3' UTR of the target mRNA (129). There is a strong preference for the 5p strand, which originates from the 5' strand of the pre-miRNA duplex (130). The strand chosen by the RISC complex is regarded as the guide strand, the strand usually with lower thermodynamic stability, while the other half of the duplex, the passenger strand, is degraded (130). Both of these strands are regarded as mature miRNAs.

Following binding to a target sequence, microRNAs can silence gene expression via translational repression or mRNA degradation (128). miRNAs, guided by RISC, target mRNA with 3' UTR complementarity to their seed sequence. GW182 helps to guide these complexes to P-Bodies where mRNA degradation often occurs (131). Once bound, the RISC can repress translation by impeding cap recognition by eIF4E and interfering with the poly(A) tail of transcripts (132). Additionally, the RISC can deadenylate and degrade their target mRNAs (133). The process of deadenylating is accomplished via binding of poly(A) Binding Protein (**PABP**) to GW182 in the RISC, which leads to recruitment and binding of Tob followed by

CAF1/CCR4 binding and deadenylation activity (134). Degradation of mRNA via miRNA targeting thus serves as a powerful regulator of gene expression.

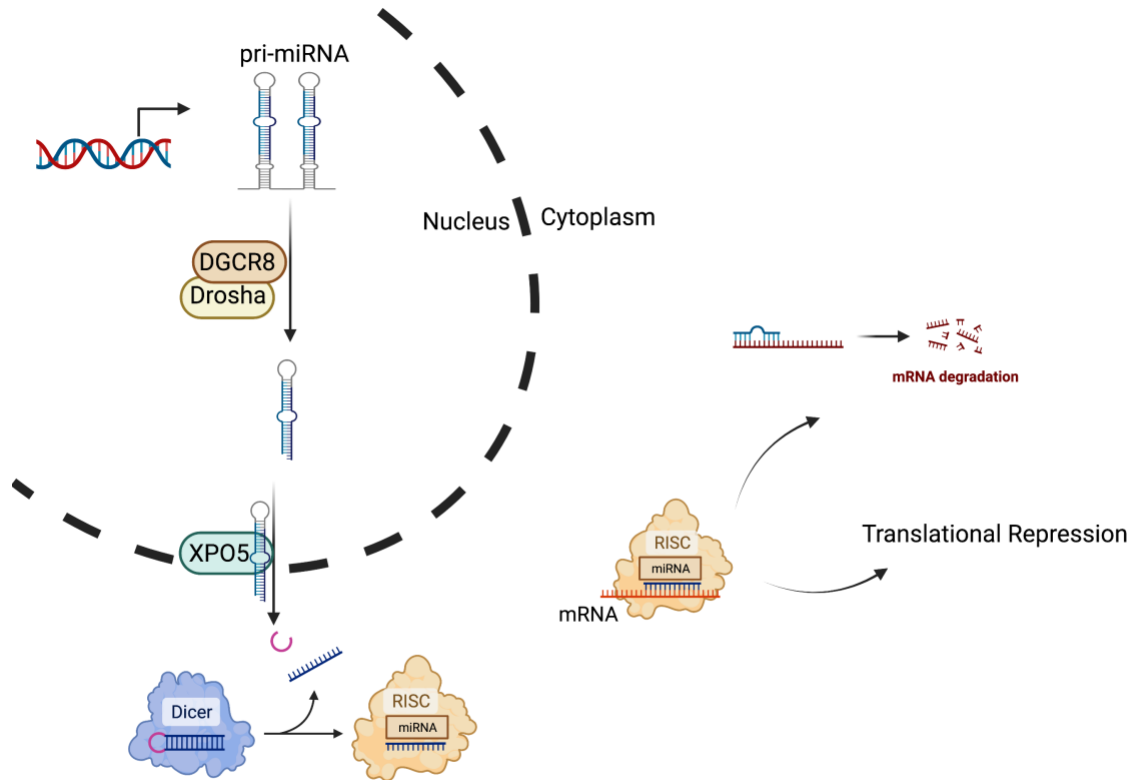


Figure 2: Schematic of miRNA Biogenesis. Pri-miRNA is transcribed and processed into pre-miRNA by the microprocessor complex. Pre-miRNA is then exported from the nucleus via XPO5 and cleaved via DICER. miRNA is loaded onto the RISC and binds to complementary sequences in the 3' UTR of a target mRNA where it translationally represses or degrades the mRNA.

miRNAs Involved in NSC self-renewal and Differentiation

Several miRNAs target genes in pathways regulating NSC self-renewal and differentiation. For example, miR-9 targets FoXO1 thus preventing the transcription of Notch

target genes and leading to differentiation (94). Further aiding in the transition to NSC proliferation, miR-9 targets repressor element-1 silencing transcription factor (REST), which maintains the NSC pool (135, 136). miR-124, on the other hand, targets Sox9 thus leading to neuroblast differentiation from TAPs (137). Likewise, let-7b overexpression leads to increased differentiation into neuroblasts and limits proliferation by targeting TLX, which controls gene expression involved in proliferation (138, 139). Moreover, let-7b targets Hmga2, which leads to increased p16Ink4 and p19Arf expression while reducing proliferative capacity of NSCs. Hmga2, in young neural stem cells fosters NSC pool maintenance via reduction in p16Ink4a expression (140).

In addition to antagonizing or maintaining the NSC pool, miRNAs aid in regulation of progenitor cell fate decisions towards neurogenesis or oligodendrogenesis. When released from neural progenitor cell exosomes, miR-21a promotes neurogenesis, while disruption of miR-21a expression in exosomes limited neurogenesis(141). On the other hand, fate determinization to oligodendrogenesis is promoted via miR-219 targeting of Sox6 and Hes5 (142, 143). Although these are just a few examples, many more miRNAs have been elucidated which alter NSC renewal or differentiation.

Aging's Impact on NSC Dysfunction and SVZ Niche Decline

Several pathways and regulatory mechanisms control neurogenesis and NSC pool maintenance, yet perturbations or alterations of these pathways and regulatory mechanisms occur with age. With age, ependymal cells in the SVZ become damaged, NSCs decline in number, and neurogenesis is markedly reduced (9, 10, 12, 144-147). The age-associated dysfunction in the SVZ niche can all be categorized into the hallmarks of aging. For the purposes of my thesis dissertation, however, I will limit the discussion to the hallmarks of stem cell exhaustion and altered intercellular communication. These hallmarks are interdependent since there are shared underlying mechanisms between them.

Stem cell exhaustion

NSCs decline in number and their self-renewal capacity with age (12, 39, 147-149). Studies of NSCs have shown that the number of activated NSCs declines by about 86% when comparing 24 month old mice to 3 month old mice (147). With the reduction in NSC number, there is also an increase in cell cycle length of NSCs when comparing 6-month-old mice to 3-month-old mice (9). With time, NSCs eventually become depleted since they shift to symmetric division (39). Therefore, with time, there is a reduction in the number of TAPs in the SVZ (9). The cause behind a reduction in TAPs is perhaps due to NSCs becoming increasingly quiescent with age to preserve the NSC pool (150). Using a combination of single-cell sequencing, computational modeling, and *in vivo* neutralizing antibody testing, Sfrp5, secreted from surrounding cells, was identified as a one of the many age-associated “gatekeeper” of quiescence. With age, it is increased in expression in mouse NSCs, and it inhibits Wnt signaling thus maintaining a quiescent state. When Sfrp5 is neutralized *in vivo*, Wnt signaling is uninhibited and leads to a shift from a quiescent to activated state in the NSCs (151). In addition to Sfrp5, FoxO3 has been identified to maintain quiescence of neural stem cells (91).

Altered intercellular communication

Several pathways of communication between cells are altered with age. FoxO signaling, for example, promotes longevity (152). Wnt signaling appears to have several alterations leading to an increasingly quiescent state in aged NSCs. In addition to the manner described in the above paragraph, Wnt signal transduction appears to be altered since p38 MAPK expression is decreased with age in NSCs and their progenitors. This reduction in p38 MAPK leads to a decline in the division of progenitors, but not NSCs themselves. The decline in proliferation is due to p38 MAPK’s diminished inhibitory effect on Dkk1, an antagonist of Wnt signaling, with age (153).

Adding to this reduction in proliferation, increased secretion of inflammatory factors, such as IL-6, with age causes activation of microglia and results in a decrease in NSC division (154). Furthermore, the number of microglia, peripheral monocytes, and CD8+ T cells increases, with age, in the SVZ, likely contributing to a pro-inflammatory environment (154, 155). Adding to these changes in cellular communication due to inflammation, astrocytes take on a reactive phenotype (156).

In addition to changes in cell-signaling due to inflammatory factors, cell-signaling through growth factors is vastly different with age. EGF and EGFR decrease with age in the SVZ resulting in a debility for NSCs to undergo neurogenesis (9, 145). Fgf2 additionally decreases with age, and if it is supplemented along with EGF in the brain of 20-month-old mice, neurogenesis increases by 170% that of a wild-type 3-month-old as demonstrated through BrdU and doublecortin (DCX) labeling (157, 158). Likewise, VEGF is shown to be decreased in the aged SVZ, but introduction of VEGF to the rat lateral ventricle helps to reduce cell death (158, 159). On the other hand, it has also been demonstrated that NSCs of 24-month-old mice, compared to 12-month-old and 3-month-old mice, are simply non-responsive to the pro-neurogenic introduction of VEGF (160). Unlike EGF, FGF2, and VEGF, IGF-1 is perhaps a more complicated story with age. As Vicario-Abejón has noted, IGF-1 signaling's effect on neurogenesis with age is different depending on the method used to study it since some common knock-out models are just deletions of certain peptides of IGF-1, so there may still be functionality (161).

miRNAs Involved in Age-Associated SVZ-Dysfunction

Although few miRNAs have been directly implicated in age-associated SVZ decline, thus highlighting the importance of a global study on the matter, some miRNAs have circumstantial evidence that they contribute to niche decline with age. The miR-106b~25 cluster has a genomic FoxO binding site for transcription, and FoxOs are tied to longevity due to their part in

insulin signaling. Likewise, miR-25 targets many genes involved in the insulin signaling pathway and BMP signaling. Knock-down of this gene limits NSC and TAP division. Increased expression of this cluster via retroviral vector in cultured NSCs leads to increased proliferation, however (162). Since FoxOs are regulated by insulin/IGF, and a reduction in insulin/IGF promotes longevity, there may be an association with this miRNA cluster and aging (93, 161, 163). Furthermore, there may be a link between aging and let-7b expression. Since let-7b increases the expression of p16Ink4a and p19Arf via targeting of Hmga2, it may be age-associated since increases in p16Ink4a and p19Arf are shown in senescence and aging (140, 164-166). Furthermore, increases in p16Ink4a have been demonstrated to reduce neurogenesis with age (166). miR-21a has been shown to promote neurogenesis in mice, yet decreased miR-21 expression is linked to poor aging in humans (141, 167). Therefore, there may be a link between this miRNA and aging as well.

In addition, there are several age-associated disease miRNAs that have function in the healthy SVZ. miR-9, miR-219a-2, and miR-124 have all been linked to Alzheimer's Disease or Multiple Sclerosis (168-171). miR-9, interestingly, showed therapeutic potential as it increases neurogenesis in the APP/PS1 mouse model of Alzheimer's Disease (170). The link of these miRNAs to healthy aging in the SVZ has yet to be explored, however. The lack of tangible links between miRNAs and neurogenesis with age stresses the need for more studies of age-associated changes in miRNA expression in the SVZ and the effects on NSC self-renewal and neurogenesis.

Sex-Specific Differences in the SVZ Impact on Aging

Unlike the hippocampus, few studies have been conducted to determine the sex-specific effect of hormones on the SVZ, as noted by Ponti et al (172). The lack of studies between both sexes is worrying since neurogenesis is affected by hormones, and the enzyme converting testosterone to estradiol, aromatase is expressed in the SVZ, and knock-out of aromatase

results in diminished ability for female mice to recognize male mice (173-175). This is important since new neurons migrate to the OB through the rostral migratory stream, and the lack of recognition of familiar males may alter reproductive behavior (173). There is conflict, however, regarding the total effect of sexual steroids on proliferation in rodents. This is due to differences in methods of changing hormones, like external exposure post ovariectomy compared to castration, and due to differences in methods for looking at proliferation of cells in the SVZ such as BrdU incorporation compared to S-phase length measurements. Furthermore, genotype plays a large role in this net effect as well. In all of these studies, however, it was clear that hormones did have a clear influence on NSC proliferation or incorporation of new neurons into the OB (172). Additionally, the role of both aging and sex, together, has been widely neglected in the field. Using rats, it was shown that NSC aging biology is sexually dimorphic. In young rats, males had greater neurogenic differentiation than females whose NSCs were more likely to commit to astrocytic lineage when stimulated with retinoic acid. With age, neuroblast number was decreased in both sexes, but it was decreased to a greater extent in female mice (176). This study, conducted in serum free media to avoid hormonal influence, shows there are innate differences in male and female aging (176). This study, in particular, emphasizes the need for more research to be conducted on innate differences in aging between the sexes in the SVZ.

Gaps in Knowledge in SVZ Aging

Since the advent of single-cell and single-nucleus technology, more studies are able to be conducted in a global, cell-type specific manner to address questions related to cell-type specific interactions and changes in the SVZ. In the past, global studies on cell-type specific gene expression within the SVZ were costly and time-consuming – involving either rigorous staining protocols that limited the amount of gene expression studied or utilized FACS to then profile the niche. Using FACS, however, biases the data since only particular cell types are sorted. Single-cell and single-nucleus RNA-sequencing technology, however, allows for the

transcriptional profiles of individual cells to be investigated without biasing cell-type. This technology has already proved useful in identifying the changing cellular composition of the SVZ with age and immune infiltration(155). Further studies will be needed, however, to determine the role of regulatory RNAs in gene-expression changes with age in each cell-type and the overall impact of that on niche decline. To this end, a reference for small regulatory RNAs, compatible with single-nucleus and single-cell technology, will need to be compiled to determine transcriptional changes. Moreover, there will need to be studies conducted on both sexes with age to characterize sexual dimorphism in each cell-type with age and the role of this on overall health and aging in the SVZ.

Impact on Human Health and Disease

By addressing the gaps in knowledge stated above, the door will open to therapeutic potentials for age-associated decline, ischemic injury, and neurodegeneration using NSCs. With age-associated decline and neurodegeneration, there are some common pathologies involving a loss of neurogenesis and brain volume loss, although neurodegenerative disease is more severe and contains additional pathologies (4, 9-11, 170, 177). In ischemic or demyelinating injury, NSC proliferation and migration has helped to repair injury (38, 76, 178). Therefore, SVZ NSCs, due to their multipotent biology, serve as the a potential molecular instrument to help repair the brain(179). On the other hand, for these therapies to be successful, the influence of age-associated gene expression in the niche environment and sexual dimorphism must be investigated, so the NSC population can survive and proliferate in a conducive capacity.

Conclusion and Significance

In this thesis, we investigate cell-type specific gene expression in both the male and female mouse SVZ niche with age. Importantly, we create a reference for pri-miRNAs that is

compatible with single-nucleus sequencing data, so pri-miRNA expression can be deduced to infer the role of miRNA expression on age-associated changes within each cell type of the SVZ niche. Furthermore, we identify changing pri-miRNAs, per cell-type, and the pathways for which their target genes are enriched. Through this analysis, we determine pri-miRNAs likely contribute to the decline of the SVZ niche by dysregulating pathways involved in cell-cell signaling necessary for NSC proliferation and self-renewal. Additionally, through this analysis, we further explore sex-specific differences in SVZ aging, and we identify pathways and genes that have sexually dimorphic expression with age. By discovering different pri-miRNA expression patterns, gene-expression patterns, and pathway ontologies in a sex-specific and cell-type specific manner, the door has opened for even more future studies regarding the overall impact sex has on aging of the SVZ niche.

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**primiReference: A Reference for Analysis of primary-microRNA Expression in Single-
Nucleus Sequencing Data**

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Abstract:

Background: Single-nucleus RNA-sequencing technology has revolutionized understanding of nuanced changes in gene expression between cell types within tissues. Unfortunately, our understanding of regulatory RNAs, such as microRNAs (miRNAs), is limited through both single-cell and single-nucleus techniques due to the short length of miRNAs in the cytoplasm and the incomplete reference of longer primary miRNA (pri-miRNA) transcripts in the nucleus.

Results: Here, we build a custom reference to align and count pri-miRNA sequences in single-nucleus data. Using young and aged SVZ (SVZ) nuclei, we have shown differences in expression of pri-miRNAs targeting genes involved in NSC differentiation in the aged SVZ. Furthermore, using wild-type and 5XFAD mouse cortex nuclei, to validate the use of primiReference, we found cell-type specific expression of pri-miRNAs known to be involved in Alzheimer's Disease.

Conclusions: pri-miRNAs likely contribute to NSC dysregulation with age and Alzheimer's Disease pathology. primiReference is paramount in capturing a more global profile of gene expression and regulation in single-nucleus data and can provide key insights into cell-type specific expression of pri-miRNAs, paving the way for future studies of regulation and pathway dysregulation. By looking at pri-miRNA abundance and transcriptional differences, regulation of gene expression by miRNAs in disease and aging can be further explored.

Key Words: primary-microRNA, Aging, subventricular zone, Alzheimer's Disease, Single-Nucleus RNA-Seq, Neurogenesis, microRNA, Transcriptomics

Background:

Adult neurogenesis occurs in 2 regions of the mammalian brain, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Neural stem cells (NSCs) in the SVZ are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes while NSCs of the hippocampus are unipotent, giving rise to neurons. With age, NSCs decline in number, self-renewal capacity, and ability to produce new neurons (1, 2). The mechanisms leading to this decline are not well understood. Within the SVZ niche, there is close interaction among the cell-types (3-5). Ependymal cells line the ventricle wall and are in close contact with the cerebrospinal fluid, while astrocytes, oligodendrocytes, NSCs, and vasculature make up the majority of SVZ. Neuronal processes can protrude into the SVZ from the striatum nearby (6, 7). NSCs have been shown to interact with surrounding cells either directly through cell to cell contact or in a paracrine fashion through secreted factors (3, 4). Due to these interactions, age-associated NSC decline could be influenced by changes occurring in the surrounding cells within the niche in addition to changes within the NSCs directly. Therefore, it is important to understand changes occurring in both the NSCs and their adjacent, interacting cell-cell types in order to obtain a comprehensive assessment of niche decline.

Over the past decade, single-cell and single-nucleus sequencing techniques have revolutionized the transcriptomics and epigenomics fields. By performing RNA sequencing (RNA-seq) on a single-cell level, scientists can identify subtle changes in gene-expression on a cell-type specific basis as opposed to the overall changes in tissue observed with bulk-RNAseq. Using single-cell, microfluidics-based techniques such as Smart-Seq and 10X Genomics, thousands of cells per tissue can be profiled. On the downside, however, tissues such as brain have cells containing long processes that can be sheared resulting in insufficient capture of the cell types. To circumvent this issue, single-nucleus sequencing technologies arose. Single-nucleus and single-cell technologies have similar, overall sensitivity, however both technologies are missing key information on small regulatory RNAs such as microRNAs (miRNAs), due to

their cytoplasmic location and small size (8, 9). To fully understand how transcriptional changes with age can influence niche decline it is necessary to include miRNA expression, since miRNAs are potent regulators of gene expression and have been shown to be involved in intercellular communication, especially in regulating neurogenesis (10-12). To address this deficit, we built a reference that can be used for single-nucleus sequencing data to discern expression of both coding genes and primary-miRNAs (pri-miRNA) sequences.

miRNAs are generated in the nucleus as pri-miRNA transcripts by RNA Polymerase II (13). The pri-miRNA transcripts are subsequently processed into pre-miRNA sequences that are exported to the cytoplasm where they are further processed into the mature miRNAs (14, 15). The mature miRNAs remain in the cytoplasm where they regulate the translation of their target mRNA transcripts. Pri-miRNAs can be monocistronic or polycistronic transcripts encoding miRNA gene sequences only, or contain protein coding and miRNA coding sequences simultaneously (15). Current single-cell sequencing technologies are unable to capture mature miRNA sequences due to their small size, which are in the range of 21-24 nucleotides (15, 16). Most pri-miRNA transcripts are several kilobases long (15-17), allowing them to be detected in single-nucleus sequencing data. However, pri-miRNAs are not currently annotated in the reference genomes for single-nucleus sequencing data. To overcome this obstacle, we combined a publicly available dataset (17) of pri-miRNA sequences with the current mouse genome annotation (mm10) to create primiReference, which can be used to identify changes in the expression of both genes and pri-miRNA sequences in single-nucleus sequencing data. Here, we used primiReference to investigate cell-type specific changes in gene and pri-miRNA expression in the SVZ isolated from young and old mice. We expand upon this data by exploring cell-type specific changes in pri-miRNA and gene expression in the cortex of the 5XFAD Alzheimer's Disease mouse model and wildtype (WT) mice (18). By including the analysis of pri-miRNA expression, we were able to gain insight into possible mechanisms of age-associated decline and Alzheimer's Disease pathology in a cell-type specific manner.

Methods:

Single-nucleus Preparation from the Male SVZ

Eight 3–4-month-old and eight 25–26-month-old C5/BL/6 male mice were sacrificed in accordance with IACUC guidelines. The SVZ was dissected from two mice and combined to constitute a sample, so this experiment had four young and four old male samples each (19). Upon dissection, we followed a modified protocol of the 10X Single-nucleus isolation protocol (10X Genomics, CG000124, Rev A) (20) **(see extended methods)**.

Single-nucleus Library Preparation and Sequencing.

The nuclei suspension was used for library preparation using the 10X v3 3' protocol (10X Genomics, CG000183, Rev A) (21). 5,000 nuclei per sample were used to construct the libraries. The libraries were sequenced to a depth of 5,000 reads per nucleus or 250 million reads per sample using the HiSeq Sequencer via Genewiz.

primiReference Construction

Fastq files from 4 young male and 4 old male samples were aligned to the mm10 genome using Cell Ranger (10X Genomics, Cell Ranger 3.0.0) (22). Resulting files merged and overlapped with a publicly available reference for pri-miRNAs in 6 cell lines (17, 23). Using a mature miRNA gff3 from miRbase and bedops (24, 25) sequences were relabeled with the mature miRNA name (26). The file was then manually inspected. The resulting GTF was appended to the mm10 GTF using 10X Cell Ranger (10X Genomics Cell Ranger 3.0.0). The mkref function was used to make a custom reference, and the Fastq files were aligned to this new 'primiReference'.

primiReference Analysis of Young and Aged SVZ

Once files were aligned and aggregated using 10X Cell Ranger, the resulting filtered feature matrices were input into Seurat (27). Nuclei were filtered, so only nuclei with features between 200 and 4384 (2 standard deviations from the mean) as well as mitochondrial contamination less than 4.06% (2 standard deviations from the mean) were subset. All these nuclei were subsequently clustered to 50 principal components, based on Jackstraw and Elbow plots, and reduced using UMap. The cells were labeled via integration with the Allen Brain Atlas and using marker genes from published literature (28-35). Astrocytes, oligodendrocytes, NSCs, and neural progenitors were subset out and differential expression analysis was conducted between the two age groups using Seurat. Following differential expression analysis, a heatmap was constructed using Morpheus (<https://software.broadinstitute.org/morpheus>) to demonstrate the significantly expressed ($p < .05$, non-parametric Wilcoxon rank sum test) pri-miRNAs within each cell type with age. These pri-miRNAs were input into miRPath using DIANATOOLS (36) using both the -3p and -5p mature sequences, since pri-miReference does not distinguish which form is expressed, and KEGG Pathways for targeted genes were determined.

Single-nucleus Sequencing of Wildtype and 5XFAD Mouse Cortex

7-month-old WT and 5XFAD samples were downloaded from GEO (GSE140511) as part of a study of single-nucleus sequencing between WT, 5XFAD, and TREM2KO groups (18). The original BAM files were downloaded then converted back to Fastqs using 10X Genomics' bamtofastq (10X Genomics, bamtofastq 1.3.2). These Fastqs were aligned using 'pri-miReference' as described above. Upon alignment and aggregation, the data was input into Seurat (27) and analyzed using the same standards as listed above for our own data. Therefore, permissible cells had between 200 and 4828.66 features and mitochondrial contamination less than 5.48% (both 2 standard deviations from the mean). The data was subsequently analyzed in the same manner as the SVZ data described above except neuronal

identities, as identified by the Allen Brain Atlas (35), were kept as defined rather than being consolidated into one neuronal class.

qPCR Validation of pri-miRNA expression in the Aged SVZ

To validate changing pri-miRNAs found using primiReference, single-nucleus data was re-analyzed as “bulk” RNA-sequencing data, so qPCR data of whole isolated SVZ can be compared to this dataset. To accomplish this, aligned primiReference BAM files of young and aged SVZ were analyzed using FeatureCounts (37) to determine the counts of each gene feature in a sample-specific rather than single-nucleus specific manner. DESeq2 (38) was then used to compare gene expression of old SVZ to young SVZ. Primers were made to pri-miR-21a and pri-miR-99a since these pri-miRNAs changed in most cell-types. RNA was extracted from five young (4 months old) and five old (23 months old) SVZ using trizol and chloroform followed by the RNEasy kit (Qiagen, #74104). RNA concentration was measured and reverse transcribed into cDNA using Taqman reagents (Thermo Fisher Scientific, #4304134, Waltham, MA). qPCR reactions were performed in triplicate. pri-miRNA expression was normalized to GAPDH, and the fold change was compared to that of the pri-miRNAs in the “bulk” analysis of the single-nucleus data. The p-value of qPCR was determined using a two-sample T test. Ggplot2 was used to plot this data (39).

Single-Cell Sequencing of Young and Aged SVZ

Using a publicly available single-cell sequencing dataset (40) of the young and aged SVZ, we determined changes in cytoplasmic mRNA levels. Single-cell sequencing data was analyzed using Seurat (27) in the same manner as our single-nucleus data. After clusters were established, differential expression was conducted between aged and young SVZ by cell-type.

Validation of Target Gene Expression in Single-Cell Data

TarBase V8 (41) was used to determine the validated targets of pri-miRNAs changing in expression within the young and aged single-nucleus SVZ data set. Using the single-cell data set of young and aged SVZ, the validated TarBase target genes were overlapped with differential expression data per cell-type. Only cell-types where the pri-miRNA was changing with age were used. The number of those target genes found in both TarBase and DE data was given as a percentage. The expression of those intersecting target genes was viewed in the single-cell data set to determine if they were increasing or decreasing in expression, with age, within that given cell-type. If the direction was the opposite of the pri-miRNA, the target was considered validated. The number of genes that were changing in the expected direction, according to pri-miRNA expression, in the single-cell data was then given as a percentage of (number of genes in expected direction/total number of target genes intersected with TarBase). These were then referred to as validated targets (**Table 2**).

Results:

primiReference Creation

To identify cell-type specific changes in miRNA expression with age in the neurogenic niche, we performed single-nucleus sequencing of the SVZ isolated from young (4-month-old) and old (25–26-month-old) mice. Single-cell and single-nucleus technologies do not allow for the capture and identification of mature miRNAs due to their small size. To circumvent this issue, we identified overlaps between SVZ data aligned to the mm10 genome and a publicly available annotation of pri- miRNAs from 6 cell lines to identify pri-miRNA transcripts in the single-nucleus sequencing data (17). We subsequently added the pri-miRNA overlaps to the mm10 genome annotation to develop a reference to identify pri-miRNA transcripts and other coding sequences (primiReference). The pri-miRNA sequences identified by primiReference overlap with the annotated pre-miRNA sequence, which is usually around 70 bp, and additional

sequence upstream and downstream (**Figure 1A**). When viewing intragenic miRNAs, the pre-miRNA sequence is often within the intron, and the pri-miRNA transcript is the unspliced transcript of the entire gene (**Figure 1B**). Accordingly, in primiReference intragenic pri-miRNAs are named as the gene name followed by the pri-miRNA name. By doing so, both gene and pri-miRNA transcript abundance is determined at once since their expression cannot be disentangled through single-nucleus sequencing. Consequently, the primiReference annotation leads to accurate mapping of pri-miRNA transcripts to the genome.

Identification of cell clusters in the SVZ

Following alignment to primiReference, we filtered the data, conducted dimensional reduction and identification of clusters (42) (**Figure 1C, Additional File 1 [Figure S1 and Figure S2], Additional File 2**). The clusters for the aging SVZ data show clear separation among the expected cell types found in the SVZ niche (**Figure 1C**). The clusters were classified using the Allen Brain Atlas initially, and markers from literature were used to further define subtypes of mural cells (pericytes and other vessel-associated subtypes), oligodendrocytes, and astrocytes (**Figure 1C**)(27-29, 31, 32, 34, 35, 43-46). The neuronal subtypes were consolidated into a single neuronal class since they were likely striatal contamination due to the lack of defined borders of the SVZ. Since our primary focus was on the NSC niche, and the neurons are underrepresented in the niche, we excluded them from the focus of this study. Furthermore, three clusters were unable to be fully defined based on the Allen Brain Atlas and literature, and these clusters, along with the choroid plexus, were excluded from the analysis.

The clusters defining the neurogenic niche - NSCs, oligodendrocyte progenitors (OPCs) and committed oligodendrocyte progenitors (COPs), mature oligodendrocytes, myelin forming oligodendrocytes (MFOs), immature neurons, neurons, microglia, and mural cells – were strongly represented in samples (**Figure 1C and Additional File 1 [Figure S2]**). Among the

cell-types, there was no bias in pri-miRNA content within each cluster as a percentage of all genes expressed (**Additional File 1 [Figure S3A]**). Likewise, there was no bias in pri-miRNA content per condition as a percentage of all genes expressed (**Additional File 1 [Figure S3B]**). These results indicate that primiReference produces accurate mapping, since primiReference was shown to have comparable expression of pri-miRNAs to coding genes. Differential expression analysis was conducted between the young and aged male mice within the cell types of the SVZ niche to gain insights into the pri-miRNAs that may contribute to the overall decline of the niche with age (**Additional File 3**).

Differential Expression of pri-miRNAs, within the Cell-Types of the Subventricular Niche, With Age:

We examined changes in pri-miRNA expression with age in the major SVZ cell types and found that each cell type had a unique signature of age-associated changes in the pri-miRNAs (**Figure 2, Table 1, Additional File 4**). While there were several pri-miRNAs that changed in most or all the cell types examined, the majority of pri-miRNAs changed in only one or a few cell types (**Additional File 1 [Figure S4A]**). Some clusters have pri-miRNAs that change only in that cluster, indicating that changes in pri-miRNA expression with age can be cell-type specific (**Additional File 1 [Figure S4A]**). While neurons, immature neurons, and pericytes had pri-miRNAs that changed with age (**Additional File 4, Table 1**), however, these will not be discussed further in this manuscript. We focused only on the pri-miRNAs that were differentially expressed in the major cell types that comprise the neurogenic niche in the SVZ.

All Cell Types

Several of the mature miRNAs encoded by the pri-miRNAs that changed globally have documented functions in the central nervous system. pri-miR-21a and pri-miR-99a decreased in all or the majority of cell types, respectively (**Figure 2**). Dysregulation of mature miR-21a

and mir-99a have been linked to impaired neurogenesis and neurodegeneration (10, 47, 48). The polycistronic transcript encoding pri-miR-1904 and pri-miR-582 increased in the majority of cell types with age (**Figure 2**). While nothing is known about the targets of the mature miR-1904, the expression of mature miR-582 has been linked to neuronal injury and inflammation (49).

Astrocytes

Two clusters of astrocytes were defined in the SVZ (**Figure 1C**), each with a distinct combination of pri-miRNAs that change with age (**Figure 2**). Overall, there were 25 pri-miRNAs that changed with age across the 2 clusters (**Figure 2, Additional File 3**), with 10 pri-miRNAs changing with age in both clusters (**Figure 2, Additional File 3**). In addition, there were 7 pri-miRNAs that changed in only in astrocyte cluster one while there were 8 pri-miRNAs that changed only in astrocyte cluster 2. Moreover, three pri-miRNAs changed only in the astrocyte clusters and not in any other cell type ((**Additional File 1 [Figure S4A]**)). The astrocyte-specific pri-miRNAs changing with age include pri-miR-3087 and pri-miR-1249, which uniquely decrease in expression, with age, within the astrocyte 1 cluster while pri-miR-6940 uniquely increases in expression, with age, in the astrocyte 2 cluster (**Additional File 1 [Figure S4A]**)).

NSCs

The changes occurring in NSC gene expression are paramount to our understanding of niche decline as a whole. Overall, eleven pri-miRNAs significantly changed in expression with age in this population (**Figure 2, Additional File 4**). One of these pri-miRNA transcripts was unique to the more “stem” populations of cells (**Table 1, Additional File 1 [Figure S4A]**). The polycistronic pri-miRNA transcript containing pri-miR-341, pri-miR-1188, and pri-miR-370 was significantly increased with age in both the NSC and oligodendrocyte progenitor and committed oligodendrocyte progenitor clusters (OPC and COP) (**Figure 2, Additional File 1 [Figure**

S4A]). The mature form of miR-370 is validated to target FoxO1, which, in combination with FoxO3, helps to control neuronal differentiation (50, 51) (**Table 1**). Furthermore the mature form of miR-341 is known to be connected to levels of REST expression (52), and it is known that REST is involved in regulating neurogenesis and alterations in REST occur in neurodegenerative conditions (**Table 1**) (53).

Oligodendrocytes

Within the SVZ, we identified several different subtypes of oligodendrocytes, including a cluster consisting of oligodendrocyte progenitor cells and committed oligodendrocyte progenitors (OPC and COPs, respectively), 2 myelin forming oligodendrocyte clusters (MFOLs), and a cluster consisting of mature oligodendrocytes (**Figure 1C, Additional File 1 [Figure S2A]**). Although all these cell-types are different sub-types of oligodendrocytes, changes in the pri-miRNA expression with age differs between the cell-types (**Figure 2, Additional File 4**). Overall, 32 pri-miRNA transcripts changed with age within these clusters. Only four pri-miRNA transcripts are found to change in in all four clusters: pri-miR-5623, pri-miR-21a, pri-miR-1936, and the polycistronic pri-miR-1904 and pri-miR-582 transcript (**Additional File 4, Figure 2**). The mature form of pri-miR-21a has been shown to be secreted into extracellular vesicles and is associated with an increase in neurogenesis (10). The mature form of pri-miR-582 is known to repress gene expression of various targets in the apoptosis pathway, and it has been associated with neuronal inflammation (49, 54).

Interestingly, pri-miR-219a-2 decreases with age in OPC and COP cluster and both MFOL clusters, but remains unchanged in mature oligodendrocytes (**Figure 2, Table 1**). OPC and COP cell-types are involved in oligodendrogenesis while MFOLs are involved in myelin formation, and these processes are greatly reduced in mature oligodendrocytes. Furthermore, these processes decrease in OPCs, COPs, and MFOLs with age. It is thus interesting that pri-

miR-219a-2, a miRNA associated with increased oligodendrogenesis and increased myelin formation is found to be decreased with age in these clusters (55-57) (**Figure 2, Table 1**).

The OPC and COP cluster contains two pri-miRNAs uniquely changing with age only within this cluster (**Additional File 1 [Figure S4A]**). pri-miR-7212 decreases with age while pri-miR-6402 increases with age (**Figure 2, Additional File 1 [Figure S4A]**).

Between the two MFOL clusters, there is much similarity in the pri-miRNAs differentially expressed with age (**Figure 2, Additional File 4**). While MFOL_1 has several more pri-miRNAs changing in expression than MFOL_2, the MFOL_1 and MFOL_2 clusters share ten pri-miRNAs significantly changing with age. Additionally, pri-miR-1251 is uniquely decreased in age within these clusters (**Additional File 1 [Figure S4A]**). Unlike the other oligodendrocyte clusters, the mature oligodendrocyte cluster does not contain any unique pri-miRNAs that change with age only in that cluster.

Validation of *primiReference* using the SVZ Single-Cell Dataset

To validate that changing pri-miRNAs lead to a functional change in mature miRNA expression, publicly available single-cell data from young and aged SVZ, which shows levels of mRNAs located in the cytoplasm where mature miRNAs are active, was used to determine if validated target genes were changing in the predicted direction (40). The single-cell data was analyzed in the same manner as single-nucleus data, and differential expression as conducted by age, per cell-type (**Additional File 1 [Figure S5]**). Using TarBase (V8) (41), to identify experimentally validated target genes for each miRNA, validated targets were overlapped with genes changing with age in the SVZ per cell-type to identify genes that were changing in the opposite direction of a pri-miRNA in expression, the percentage of these genes was noted as the percent of validated genes for our *primiReference*. For example, pri-miR21a decreased in expression in all cell-types, mmu-miR-21a-3p targets were overlapped with differential expression data from each cell type. Interestingly, 60-86% of the targets that were differentially

expressed in the single-cell data set and validated as mir-21a targets in TarBase were changing in the predicted direction, suggesting that the change in pri-miRNA expression in the nucleus may lead to a corresponding change in the mature miRNA expression in the cytoplasm. For all pri-miRNAs that had experimentally validated targets, an average of 72% of those validated target genes that were differentially expressed changed in the predicted direction by pri-miRNA expression (**Table 2**).

To validate the changes in expression in pri-miRNAs found in our data, qPCR was conducted on pri-miR-99a and pri-miR-21a since these pri-miRNAs were decreasing in most cell-types. To have a fair comparison of expression levels between whole isolated SVZ in qPCR data and our single-nucleus dataset, the single-nucleus data was combined to form “bulk” RNA-sequencing data, so that cell-type specific expression was eliminated and only expression of total reads in old SVZ compared to young SVZ is shown (**Additional File 5, Figure 3A, B**). In bulk sequencing data, pri-miR-21a changes by -0.77 in old mice while in qPCR data it is shown to decrease -0.67 ($p < .05$). Meanwhile, pri-miR-99a loses its differential expression when analyzed in bulk sequencing data and the same was shown in qPCR data (**Figure 3A, Figure 3B**). This underscores the validity of pri-miReference as well as highlights single-nucleus sequencing’s ability to pick up on subtle changes in gene-expression within cell-types that are not shown in bulk-sequencing.

Pathways of Predicted Target Genes of pri-miRNAs Differentially Expressed in Aged SVZ Compared to Young SVZ

To determine the possible physiological consequences of the changes in expression of the pri-miRNAs elucidated in this study, mirPath (36) was used to determine which pathways may be affected by changes in predicted target genes of the mature sequences of the differentially expressed pri-miRNA for each cell type (**Figure 3C, Additional File 1 [Figure S4B], Additional File 6**). As expected, each cell type had a unique set of pathways that may

be affected by the changing pri-miRNAs. Overall, pathways involved in cell proliferation, survival, and cancer as well as pathways involved in development and differentiation, calcium signaling, and cell-cell interaction were predicted to be affected by the combination of changing pri-miRNAs with age in the SVZ (**Figure 3C, Additional File 6**). MAPK signaling, Wnt signaling, FoxO signaling, and Hippo signaling were enriched for predicted target genes of pri-miRNAs differentially expressed with age in every cell-type. This is interesting, since all these pathways regulate development and differentiation. Intriguingly, signaling pathways regulating pluripotency of stem cells was enriched in all cell-types except the ependymal cells and neurons (**Figure 3C**).

Several of the differentially expressed pri-miRNAs elucidated from astrocyte clusters have validated target genes (**Table 1**). Astrocyte cluster 2 has increased expression of pri-miR-137, the mature sequence of which represses targets of Msi1, which are involved in maintaining stemness (58) (**Table 1**). Astrocyte clusters 1 and 2 have increased expression of pri-miR-128-2 and the mature sequence targets stem cell factors KLF4, CSF1, and SNAI1, which help maintain stemness (59).

Most pri-miRNAs that change with age in the astrocyte clusters do not have experimentally validated target genes. miRPath predicts pathways largely involved in cell proliferation, survival, and cancer as well as development and differentiation, calcium signaling, and cell-cell interaction with the most predicted target gene enrichment in pathways in cancer, proteoglycans in cancer, PI3K-AKT signaling, MAPK signaling, Wnt signaling, Hippo signaling, and FoxO signaling from these classes (**Figure 3C, Additional File 6**). Meanwhile, the pathways of predicted target genes of uniquely expressed pri-miRNAs in the first astrocyte cluster largely fell into metabolic or cell proliferation, survival, and cancer pathways.

In the NSC cluster, most predicted target genes belong to pathways involved in cell proliferation, survival, and cancer as well as CNS pathways and pathways involved with differentiation and development. NSC show differential expression of pri-miRNAs targeting

genes involved in FoxO signaling, TGF β signaling, signaling of pathways regulating pluripotency of stem cells, MAPK signaling, Wnt signaling, and Hippo signaling. Interestingly, 24 predicted target genes belong to the TNF signaling pathway (**Figure 3C, Additional File 6**). Furthermore, NSCs have increased expression of pri-miR-490, and the mature sequence of which is known to target HDAC2 expression (60). Manipulating HDACs has been shown to dysregulate neurogenesis (61). Together, these data demonstrate that changing pri-miRNA expression in the aged NSCs may impact the pathways regulating their differentiation.

Oligodendrocyte progenitors have differentially expressed pri-miRNAs that are predicted to target genes that are largely associated with pathways involved in development and differentiation, as well as cell-cell interaction, similar to the NSCs. MAPK signaling, Wnt signaling, FoxO signaling, Hippo signaling, signaling pathways regulating pluripotency of stem cells, and TGF β signaling are all enriched for predicted target genes of pri-miRNAs changing with age in the OPCs (**Figure 3C, Additional File 6**). These pri-miRNAs may play into the dysfunction in oligodendrogenesis observed with age.

The two MFOL clusters have differentially expressed pri-miRNAs that are predicted to target genes involved in pathways associated with development and differentiation, similar to the OPC cluster (**Figure 3C, Additional File 6**). Interestingly, the MFOL_1 cluster has predicted target genes in the fatty acid biosynthesis pathway (**Additional File 6**). Fatty-acid biosynthesis is essential for myelin formation.

Although the mature oligodendrocyte cluster does not contain any unique pri-miRNAs that change with age, the pathways belonging to predicted target genes of the distinct combination of increased pri-miRNA expression with age are broad, and a few are novel to this subtype of oligodendrocyte (**Figure 3C**). The pathways enriched for predicted target genes are involved in development and differentiation and CNS signaling. Of note, the Amyotrophic Lateral Sclerosis pathway is enriched for predicted target genes of pri-miRNAs changing with age in the mature oligodendrocyte cluster (**Figure 3C, Additional File 6**). Furthermore, pri-miR-

384 increases in expression, and the mature form of pri-miR-384 is known to target BACE1 (62). BACE1 cleaves amyloid precursor protein and thus plays an important role in Alzheimer's Disease pathology (63).

In microglia, pathways containing the predicted target genes of the differentially expressed pri-miRNAs are heavily enriched for pathways involved in development and cell differentiation (**Figure 3C, Additional File 6**). As expected, microglia have the greatest number of predicted target genes involved in the immune response. Thirty-six predicted target genes are involved in mRNA surveillance pathways while 46 predicted target genes are involved in JAK-STAT signaling (**Additional File 6**). Changes in these pathways are significant since microglia play a crucial role in the immune response in the brain.

Analysis of wildtype and 5XFAD mouse cortex aligned to primiReference

To validate the utility of the reference in working with different single-nucleus datasets, we used primiReference to characterize the pri-miRNA and gene expression profiles in the cortex of wild-type and the 5XFAD Alzheimer's Disease mouse model using a publicly available single-nucleus sequencing dataset (18). As seen through the UMap, there were far more clusters of neurons and a greater diversity of neurons as expected from the cortex (**Figure 4A**). Moreover, different layers of cortical interneurons were able to be identified. Like the SVZ, there was no bias in pri-miRNA content within each cluster as a percentage of all genes expressed (**Additional File 1 [Figure S3C]**). Additionally, there was no significant difference in the pri-miRNA content per condition as a percentage of all genes expressed (**Additional File 1 [Figure S3D]**). Since primiReference did not bias clustering based on cell identity, nor did primiReference uncover bias in overall pri-miRNA expression between cell-types in conditions, the cortex was further analyzed to gain insights into 5XFAD associated pri-miRNA changes within each cell type (**Figure 4B, Additional File 1 [Figure S6], Additional File 7**).

Differential expression of pri-miRNAs within the cortex of 5XFAD mice compared to wildtype mice

All Cell-Types

As seen in the SVZ data, each cell type has a unique profile of pri-miRNAs that change in expression in the 5XFAD data (**Figure 4B**). More pri-miRNAs were increased in expression than decreased in expression, as a whole, in the 5XFAD condition compared to wild-type (**Figure 4B, Additional File 8**). Interestingly, there were no pri-miRNAs that changed in all cell-types. These data indicate that changes in pri-miRNA expression in the 5XFAD model are cell-type specific.

Neurons

As expected from cortex data, there were more distinct populations of neurons identified in this dataset (**Figure 4A**). These distinct populations were analyzed individually, instead of consolidating the clusters into one neuronal cell type as was done for the SVZ dataset, since it is important to understand the differences in the pri-miRNA expression in the different subtypes of neurons in the cortex in the context of Alzheimer's Disease. There are fourteen subtypes of neurons identified in the cortex data, using the Allen Brain Atlas and the literature (**Figure 4A, (Additional File 1 [Figure S2B]), Additional File 7**).

The neuronal subtypes had large diversity in differential expression of pri-miRNA differences in the 5XFAD model. As expected, pri-miR-219a-2 and pri-miR-124 were both found to decrease in expression within certain cell types in the 5XFAD condition, since these were shown to decrease in Alzheimer's Disease (64-66) (**Figure 4B, Table 1**). pri-miR-219a-2 decreases in expression in the L6b, L6 CT, L6 IT, L5 PT, L5 IT, L2/3 IT, and Meis2 clusters while pri-miR-124 decreases in expression in L5 PT, NP, PValb, Lamp5, immature neurons, astrocyte 1, OPC 1, all 3 MFOL clusters, and the VLNC cluster (**Figure 4B**). These data show that changes in pri-miRNA expression is cell-type specific in the 5XFAD model, suggesting that

this is also true in Alzheimer's disease in humans. Although few pri-miRNAs found in this dataset have experimentally validated target genes, it is validated that the mature sequence of pri-miR-3078, which is co-transcribed with pri-miR-124-1, is found to decrease in APP/PS1 mice compared to wild-type mice thus showing consistency among Alzheimer's models (67) (**Table 1**).

Astrocytes

Three clusters of astrocytes were defined in this dataset (**Figure 4A**). In total, 13 pri-miRNAs change amongst these clusters in the 5XFAD condition compared to wildtype. All three astrocyte clusters only have one pri-miRNA transcript changing in the 5XFAD condition compared to wildtype in common: the polycistronic pri-miR-7078, pri-miR-7077 transcript. Astrocyte clusters 1 and 2 both have an increase in expression of pri-miR-1903 as well. On the other hand, astrocyte clusters 2 and 3 have an increase in expression of pri-miR-7117 in the 5XFAD condition while astrocyte cluster 1 does not (**Figure 4B**). Astrocyte cluster 3 has unique changing expression of pri-miR-3075, which does not significantly change in any other cell-type in the 5XFAD condition compared to wild-type (**Additional File 1 [Figure S7A]**).

Overall, few of the mature miRNAs encoded by the pri-miRNAs elucidated in this study are a part of the scientific literature. Of those that are, miR-124-1, which is decreased in expression in the astrocyte 1 cluster, is known to decrease in expression in Alzheimer's patients, and it is validated to target BACE1, which is heavily involved in Alzheimer's pathology (66, 68) (**Table 1**). The pri-miRNA sequence for miR-219a-2 decreases in the astrocyte 2 cluster, and miR-219a-2 has been shown to regulate the tau pathway (65) (**Table 1**).

Oligodendrocytes

There were several additional distinct clusters of oligodendrocyte cell types defined in the cortex than in the aging SVZ (**Additional File 1 [Figure S2B]**). There are three OPC, one

COP, three MFOL, and one mature oligodendrocyte clusters (**Figure 4A**). Overall, 11 pri-miRNAs changed in expression in the 5XFAD condition compared to wildtype in then OPCs, while 18 pri-miRNAs changed in all oligodendrocyte clusters, with no single pri-miRNA changing only in the oligodendrocyte clusters (**Figure 4B, Additional File 8**). Intriguingly, pri-miR-219a-2 is decreased in expression in the only the second of the OPC clusters rather than all three. This is interesting since decreased miR-219a-2 expression is known to limit oligodendrogenesis (56, 69) (**Table 1**).

Distinct from the OPC clusters, the COP cluster contains only 2 pri-miRNAs that are changed in the 5XFAD model. One pri-miRNA transcript increasing in expression in the 5XFAD model, the polycistronic pri-miR-7078, pri-miR-7077, and one pri-miRNA decreasing in expression in the 5XFAD condition, pri-miR-6953 (**Figure 4B**). Like the COP cluster, the mature oligodendrocyte cluster only has one pri-miRNA increasing in expression in the 5XFAD condition, pri-miR-8104, and one pri-miRNA decreasing in expression in the 5XFAD condition, pri-miR-1251.

Among the myelin forming oligodendrocytes (MFOLs), there are a total of 12 pri-miRNAs changing in the 5XFAD condition (**Figure 4B**). Interestingly, all three MFOL clusters each have a pri-miRNA changing in expression within their cluster that is unique to that cluster (**Additional File 1 [Figure S7A]**). MFOL 1 has a decrease in expression of pri-miR-490 in the 5XFAD condition, which is validated to target HDAC2 (60) (**Table 1**). MFOL 2 has a decrease in expression of pri-miR-124-2 in the 5XFAD condition while MFOL 3 has an increase in expression of pri-miR-3092 in the 5XFAD condition (**Additional File 1 [Figure S7A]**). pri-miR-219a-2 is found to have decreased expression in the MFOL 3 cluster (**Table 1**). While the mature sequences of few pri-miRNAs have validated target genes, decreased miR-219a-2 is validated to decrease myelination and be involved in Multiple Sclerosis and Alzheimer's Disease (57, 69, 70).

Pathways of Predicted Target Genes of pri-miRNAs Changing in the 5XFAD Cortex Compared to Wild-Type

The pathways affected by the predicted target genes of the mature sequences of the pri-miRNAs differentially expressed in the 5XFAD model show a unique pattern for each cell-type (**Figure 5**). There is no pathway that has enrichment of predicted target genes in all cell-types. This analysis shows the diversity of miRNA targeting in the cortex in the 5XFAD condition compared to wild-type.

Interestingly, there are a number of pathways predicted to be affected by the changing pri-miRNAs in the 5XFAD model that are associated with neurodegenerative conditions. Among the L6 neurons, there is enrichment in pathways with predicted target genes in the glycosphingolipid biosynthesis – lacto and neolacto series pathway (**Figure 5, Additional File 9**). Alterations in glycosphingolipid metabolism have been found to cause neuroinflammation in Parkinson's Disease (71). Furthermore, many neuronal subtypes have predicted target genes within the axon guidance pathway. Interestingly, pri-miRNAs differentially expressed in the L5 PT, NP, Pvalb, Lamp5, and immature neurons in the 5XFAD condition have predicted target genes enriched for prion disease (**Figure 5, Additional File 9**). Prion protein has been implicated in Alzheimer's Disease and serves as a potential therapeutic target (72, 73). L5 PT neurons additionally have enrichment of predicted target genes for several pathways involved in cell-cell interaction (**Figure 5, Additional File 9**). On the other hand, pri-miRNAs that are differentially expressed in the 5XFAD condition in L2/3 IT and Lamp5 neurons largely target genes that fall into CNS signaling pathways and metabolic pathways (**Figure 5, Additional File 9**). There is a wide diversity among enriched pathways among neuronal subtypes, supporting the idea that the 5XFAD mutations promoting the development of AD pathology affect pri-miRNA expression in a cell-type specific manner.

All three astrocyte clusters share common pathways involved in development and differentiation, CNS signaling, and cell-cell interactions, that are predicted to be affected by the changes in pri-miRNA expression (**Figure 5, Additional File 9**). However, there are unique pathways found in each cluster. The first astrocyte cluster has enrichment for prion diseases, RNA degradation, cell cycle, focal adhesion, and regulation of actin cytoskeleton pathways, that are not seen in the other two astrocyte clusters. The second astrocyte cluster has more CNS pathways involved in reward and addiction such as morphine addiction, cocaine addiction, and circadian entrainment. The third cluster includes unique pathways involved in development and differentiation such as Hippo signaling and signaling pathways regulating pluripotency of stem cells (**Figure 5, Additional File 9**). These data demonstrate that although there are many common overlaps between all three astrocyte clusters in their pri-miRNA expression and predicted target genes, there is additional variability between them likely contributing to functional differences.

The oligodendrocyte clusters also show diversity in the pathways affected by changing pri-miRNAs in the 5XFAD model. (**Figure 5, Additional File 9**). Only the first OPC cluster has predicted target genes belonging to pathways involved in cell-to-cell interactions in the 5XFAD condition compared to wild-type (**Figure 5**). The second OPC cluster has predicted target genes belonging to CNS related pathways. In the COP cluster, however, the mature sequences of the only two changing pri-miRNAs, are predicted to target genes involved in development and differentiation and metabolism (**Figure 5, Additional File 9**).

For the MFOL clusters, pathways belong to development and differentiation and CNS signaling pathways are enriched in all 3 clusters. However, some pathways are represented in just one of the MFOL clusters, including the 21 predicted target genes of pri-miRNAs changing in expression in the MFOL 2 cluster in the 5XFAD model belonging to the RNA degradation pathway (**Figure 5**). These results indicate that changes induced by differential pri-miRNA

expression in the oligodendrocyte clusters are cell-type specific and have different consequences in different cell-types.

Discussion:

Currently, mature miRNAs are undetectable in single-cell data due to size exclusions used during sequencing library preparation, and pri-miRNAs are undetectable in single-nucleus data due to a lack of annotated reference. Since miRNAs serve as potent regulators of gene-expression, the inability for these transcripts to be detected on a single-nucleus/single-cell level is a great limitation to all fields utilizing this -omics technique. By creating an annotation of pri-miRNAs for single-nucleus sequencing data, this tool and strategy will now allow for novel insights into cell-type specific expression of pri-miRNA species.

Previously, only small-RNA-sequencing data was able to yield differential expression data of miRNAs via sequencing. This technique, however, was limited since information about gene expression was obtained from separate RNA-sequencing data. With use of *primiReference*, gene and pri-miRNA transcripts are analyzed within the same dataset. In addition, small RNA sequencing data had to be conducted in “bulk” – RNA sequencing libraries created from RNA isolated from whole tissues. Current single-cell and single-nucleus protocols do not capture small RNAs, meaning that there was no information about small RNA expression in individual cell types. With the development of single-nucleus sequencing, data can be derived about pri-miRNA expression on a cell-type specific basis. It also allows for discernment of changes in expression that may occur in only a small subset of cells that would have otherwise masked in bulk RNA-seq data.

To discover differential expression of pri-miRNAs with age in the brain, a neurogenic region of the brain, the SVZ, was selected. The SVZ contains several cell types, including NSCs, astrocytes, oligodendrocytes, neurons, ependymal cells, and mural cells. Since this region is neurogenic, there are also diverse subtypes of progenitors, since the NSCs are

multipotent and can differentiate into astrocytes, neurons, and oligodendrocytes. Understanding cell-type specific differences in the SVZ gives insight into the functioning of the neurogenic niche, which relies heavily on cell-cell signaling (74). Since miRNAs are highly involved in regulation of development and maturation of cell types, it was vital to explore differences in pri-miRNAs with age in this region. Additionally, the publicly available single-nucleus data set on wild-type and 5XFAD mice was analyzed using the same workflow to determine the differential pri-miRNA expression in an Alzheimer's disease model in a cell-type specific manner.

Through our analyses using priReference, we found several differentially expressed pri-miRNAs that may aid in our overall understanding of NSC decline within the subventricular niche and decline as a whole. For example, pri-miR-21a is decreased with age in NSCs, and in other cell-types. An increase in miR-21a is associated with increased neurogenesis and a decrease in this miRNA is associated with aging (10, 75). Moreover, miR-21a is predicted to target SIRT1. A reduction of miR-21a could lead to an increased SIRT1 expression, and increased SIRT1 has been shown to increase in NSC differentiation into astrocytes as opposed to neurons, which is a hallmark of the aging SVZ niche (1, 76). Therefore, the decreased expression of this pri-miRNA could contribute to the decrease in neurogenesis within the SVZ with age (10).

The predicted mRNA targets of all pri-miRNAs differentially expressed with age in the SVZ fell into pathways associated with development and differentiation such as Hippo signaling, signaling pathways regulating pluripotency of stem cells, FoxO signaling, and Wnt signaling. There is likely dysregulation of these pathways, and this may contribute to NSC exhaustion with age (1, 2). Furthermore, pri-miR-490, pri-miR-128-2, pri-miR-341, and pri-miR-370 were increased in expression in aged SVZ NSCs and have all been implicated in decreasing NSC proliferation or commitment to neural progenitors (50, 51, 53, 59-61, 77). Therefore, the increased expression of these miRNAs could contribute to decreased neurogenesis with age.

Taken together, the validated and predicted targets of the pri-miRNAs changing in NSCs with age in the SVZ could contribute to a reduction in neurogenesis.

Astrocytes in the SVZ play a significant role in regulating NSC differentiation and proliferation through secretion of factors involved in Notch, Wnt, and Shh signaling (3, 78). The two astrocyte clusters had pri-miRNAs differentially expressed with age that are predicted to target genes in pathways associated with NSC differentiation and proliferation. The predicted targets of all pri-miRNAs differentially expressed with age in the astrocyte clusters belong to pathways associated with development and differentiation such as Hippo signaling, signaling pathways regulating pluripotency of stem cells, FoxO signaling, and Wnt signaling. Dysregulated expression of some of the predicted target genes of these pathways resulting from altered miRNA expression may lead to differences in NSC differentiation or proliferation, since signaling proteins like Bmp7 and Wnt3 are predicted target genes.

The dysregulation of neurogenesis is not limited to the NSCs and astrocytes. Immature neurons from the SVZ have decreased expression of pri-miRNA cluster pri-miR-24-1, 27b, 23b. Knockout of these pri-miRNAs caused a decrease in glycolytic byproducts, indicating a decrease in glycolysis (79). When immature neurons are undergoing the maturation stages, they rely mainly on glycolysis for energy (80). Therefore, an impairment in glycolysis would likely hamper the ability for immature neurons to reach maturity. Furthermore, evidence of impaired, incomplete neuronal formation is found in the differential expression of pri-miRNAs in the oligodendrocytes. A decrease in pri-miR-219a-2, with age, is found in both myelin forming oligodendrocyte clusters as well as the OPC and COP cluster, and a decrease in this miRNA is associated with decreased myelination and re-myelination of neurons (57, 70). Additionally, decreased miR-219a-2 expression is implicated in impaired oligodendrogenesis of OPCs (55, 56).

Along with impaired neurogenesis, some pri-miRNAs may be involved in immune modification. pri-miRNA-6394 is predicted to target the enzyme Neu1, which degrades sialo-

modifications, and St6galnac, which catalyzes the transfer of sialic acid residues (81). Interestingly, sialylation has implications in neurodegeneration and neuroinflammation since a reduction in sialylation leads to inappropriate phagocytosis of neurons by activated microglia in an inflammatory environment, contributing to neuronal loss (81). Moreover, microglia, along with the OPC and COPs, have decreased expression of pri-miRNAs with predicted targets in the cytokine-cytokine signaling pathway, indicating a potential dysregulation of cytokine production. Meanwhile, NSCs have decreased pri-miRNA expression of six different pri-miRNAs with predicted target mRNAs in the TNF signaling pathway including Cxcl2, Cxcl1, Ikbkb. Additionally, pri-miRNAs decreasing with age in the astrocyte clusters with age are predicted to target Ikbkb as well. These results indicate that changes in pri-miRNA expression could lead to neuroinflammation with age and in Alzheimer's disease.

Together, our data indicates that several pri-miRNAs, changing with age, have either been demonstrated to target pathways involved in neurogenesis, oligodendrogenesis, myelination, and immature neuron maturation or are predicted to target genes in pathways which regulate these processes. Additionally, our data suggests that several pri-miRNAs that are differentially expressed in the aged SVZ are predicted to target pro-inflammatory cytokines and processes. Many miRNAs do not have validated target genes, and thus experimental studies on the predicted target genes and their respective pathways is needed to truly understand the breadth of miRNA impact on the aging subventricular niche.

Many of the miRNAs differentially expressed in the 5XFAD cortex compared to the wild-type cortex do not have any published data on their targets. On the other hand, those that have validated target genes are highly implicated in the pathology of Alzheimer's disease, thus demonstrating the utility of this tool to narrow down which cell types specifically play a role in the development of the disease. pri-miR-124-1 is decreased in expression in certain subpopulations of neurons, immature neurons, astrocytes, oligodendrocyte progenitors, committed oligodendrocyte progenitors, mature oligodendrocytes, and immune cells in the

5XFAD mice. miR-124 is known to decrease in expression in the brains of Alzheimer's patients, and this miRNA targets BACE1, which is directly involved the development of the amyloid pathology of Alzheimer's Disease (66, 68). Furthermore, pri-miR-3078 decreases in expression in both 5XFAD models and APP/PS1 models of Alzheimer's disease (67). Interestingly, one of the predicted target genes, which would be increased in expression with the reduction of miR-3078, is *Prnp*. *Prnp* is implicated in behavioral insufficiencies seen in Alzheimer's Disease pathology (73).

Similar to the aging SVZ, pri-miR219a-2 and pri-miR-338 were both decreased in the 5XFAD cortex. Decreased miR-219a-2 expression is implicated in neurodegenerative diseases such as Alzheimer's Disease and Multiple Sclerosis (69). In Alzheimer's Disease, miR-219a-2 additionally plays a role in Alzheimer's pathology since this miRNA can regulate tau expression (65). Furthermore, Alzheimer's Disease is characterized by demyelination and myelin damage, while deficits in miR-219a-2 and miR-338 cause impaired myelination and re-myelination (57, 82). Therefore, by understanding which cell types have deficits in these pri-miRNAs, targeted therapeutic strategies can be developed to alleviate the pathology caused by their reduction, or targeted therapeutic strategies can be developed to artificially increase their expression.

Of importance, this study highlighted the specificity of each neuronal subtype in terms of differential expression of pri-miRNAs in the 5XFAD condition compared to wild-type. Neurons are often treated as a group rather than by individual subtype in studies. Computational analysis of these different subpopulations highlights that the 5 familial AD mutations caused significantly different changes in differential expression of pri-miRNAs among the neuronal subtypes, providing insights for future studies to understand the impact of these changes on each subtype and their contributions to AD development.

Using the priReference tool, cell-type specific changes in pri-miRNA expression within the aging SVZ and 5XFAD mouse cortex were elucidated. With these findings, new cell-type specific targets for neurogenesis are now available for study as well as new-cell type specific

targets for Alzheimer's Disease. By investigating the pri-miRNAs found through the primiReference tool, new avenues of research into manipulation of pri-miRNAs and their mature sequence can be conducted in a more targeted, cell-type specific manner, which may help mitigate neuropathology.

Conclusions:

In conclusion, we developed a novel reference for use in single-nucleus data to derive expression data of pri-miRNAs. Through this reference, we identified differentially expressed pri-miRNAs in our own data set of young and aged SVZ which may contribute to NSC and niche decline. We have additionally found cell-type specific differential expression of pri-miRNAs implicated in Alzheimer's Disease pathology in the 5XFAD cortex data set. By determining the genes and pathways that these miRNAs target, in a cell-type specific manner, we will be able to gain new, more informative insights when conducting pathway analysis in single-nucleus datasets. Together, primiReference will help to identify novel targets for disease and other conditions, such as aging, by determining differential expression of pri-miRNAs on a cell-type specific basis.

Table 1: Function of Mature miRNAs Processed From pri-miRNA Transcripts Differentially Expressed Within the Aging SVZ and 5XFAD Cortex

miRNA	Cell types with DE of the pri-miRNA transcript in Aged SVZ compared to Young SVZ	Expression of the pri-miRNA transcript in 5XFAD Cortex compared to Wild-Type	Cellular Processes and Gene Targets
miR-21a	↓ Expression: All cell types	NA	Increases neurogenesis (10), found to be associated with the aging mouse (75)
miR-99a	↓ Expression: Neuron, Immature Neuron, Astrocyte_1, Astrocyte_2, NSC,	NA	Decreases mTOR expression (48). Likely involvement in commitment of progenitors to glial lineage (47).

	OPCandCOP, MFOL_1, MFOL_2, Microglia, Immune Cells, Vascular Endothelium		
miR-24-1	↓ Expression: Immature Neuron	NA	Knockout of this cluster leads to decreased NAD and other glycolytic metabolites (79). Impaired glycolysis prevents neuronal differentiation and maturation (80). Furthermore, this cluster is increased in terminally differentiated cells (83).
miR-27b	↓ Expression: Immature Neuron	NA	Mir27b targets AMPK (84). Increases in AMPK are associated with aged hippocampal NSCs and their decline (85). Knockout of this cluster leads to decreased NAD and other glycolytic metabolites (79). Impaired glycolysis prevents neuronal differentiation and maturation (80). Furthermore, this cluster is increased in terminally differentiated cells (83).
miR-23b	↓ Expression: Immature Neuron	NA	Knockout of this cluster leads to decreased NAD and other glycolytic metabolites (79). Impaired glycolysis prevents neuronal differentiation and maturation (80). Furthermore, this cluster is increased in terminally differentiated cells (83).
miR-219a-2	↓ Expression: Astrocyte_1, Astrocyte_2, OPCandCOP, MFOL_1, MFOL_2, Microglia, Ependymal, Vascular Endothelium	↓ Expression: L6b, L6 CT, L6 IT, L5 PT, L5 IT, L2/3 IT, Meis2, Astrocyte_2, OPC_2, MFOL_3	Increases myelination and re-myelination as well as increasing OPC differentiation (55-57, 70). Decreased levels have been described in neurodegenerative diseases such as MS and AD (69). In AD models, it has been shown that miR-219a can regulate tau expression (65).
miR-338	↓ Expression: Astrocyte_1, Astrocyte_2, NSC, Ependymal Cell, Vascular Endothelium	NA	Knockout of miR-338 causes a worsened phenotype of dysregulated myelination of miR219 mutants (57). These miRNAs work together to induce proper myelination.
miR-137	↑ Expression: Neuron, Immature Neuron, Astrocyte_2, OPCandCOP, Vascular Endothelium	NA	Represses Msi1 and thus leads to stem cell differentiation (58). Additionally targets Src and thus leads to neuroprotection from secreted inflammatory cytokines and proteins (86).
miR-582	↑ Expression: Neuron, Immature	NA	Represses various target genes in the apoptosis pathway (54).

	Neuron, NSC, OPC and COP, MFOL_1, MFOL_2, Mature Oligodendrocyte, Microglia, Immune Cells, Pericyte, Ependymal Cell, Vascular Endothelium		
miR-490	↑ Expression: Neuron, NSC, Ependymal Cell	↓ Expression: MFOL_1	Directly inhibits HDAC2 (60), which is involved in switching from a stem to a committed progenitor cell (61).
miR-128-2	↑ Expression: Astrocyte_1, Astrocyte_2, NSC, MFOL_1, Mature Oligodendrocyte, Microglia	NA	Directly targets stem cell factors such as KLF4, CSF1, NANOG, and SNAIL (59). Furthermore, it has been shown that this miRNA reduced NPC proliferation (77).
miR-218-2	↑ Expression: Astrocyte_2, MFOL_1, Microglia, Immune Cells, Ependymal Cells, Vascular Endothelium	NA	Increased expression in human Alzheimer's Disease patients has been observed (87). Additionally, this miRNA can alter the Wnt signaling pathway leading to differentiation - an important part of neurogenesis (88).
miR-384	↑ Expression: Mature Oligodendrocyte, Microglia, Vascular Endothelium	NA	Targets BACE1 expression involved in Alzheimer's Disease (62).
miR-341	↑ Expression: NSC, OPC and COP	NA	KO of REST led to increased expression of this miRNA (52) indicating that is likely involved in NSC differentiation since REST expression promotes stemness (53)
miR-370	↑ Expression: NSC, OPC and COP	NA	miR-370 targets FOXO1 (50), which - in combination with FOXO3, helps to control autophagic flux with neuronal differentiation (51).
miR-128-1	↑ Expression: Pericyte	NA	miR-128-1 targets IGF1 (89).
miR-124-1	NA	↓ Expression: L5 PT, NP, PValb, Lamp5, Immature Neuron, Astrocyte_1, OPC_1, MFOL_1,	Targets BACE1 expression involved in Alzheimer's Disease (66), and mir-124 was found to be decreased in the brains of AD patients (68). Additionally, the gene Drd2 is targeted by miR-124-1 and thus the decreased miR-124-1 expression decreases prefrontal cortex performance (90).

		MFOL_2, MFOL_3, VLMC	
miR-3078	NA	↓ Expression: L5 PT, NP, PValb, Lamp5, Immature Neuron, Astrocyte_1, OPC_1, MFOL_1, MFOL_2, MFOL_3, VLMC	Decreased in APP/PS1 mice compared to controls (67).

Table 1: Function of Mature miRNAs Processed From pri-miRNA Transcripts Differentially Expressed Within the Aging SVZ and 5XFAD Cortex: Column 1 indicates the mature miRNA name for pri-miRNAs that are differentially expressed with age in the SVZ or in the 5XFAD compared to wild-type in mouse cortex, with validated target genes in the literature. Column two indicates the direction of miRNA differential expression as well as which cell-types are differentially expressing these miRNAs in the SVZ with age. Column three indicates the direction and cell-type of differentially expressed miRNA in the 5XFAD cortex compared to wild-type. Column 4 describes relevant literature on the miRNA.

Table 2: Overlap of Tarbase validated target genes of miRNAs and expression of cell-type specific targets in single-cell SVZ data

miRNA	Cell-Type	Number of Targets Present in Cell-Type Expression Data	Percentage of Targets Validated
miR-21a-3p	Neuron	20	65%
	Astrocyte	22	64%
	qNSC	20	60%
	aNSC1	25	72%
	aNSC2	22	86%
	MFOL	28	61%
	MOL	24	75%
	Microglia	25	80%
	Pericyte	21	76%
	Vascular Endothelium	16	81%

miR-338	Astrocyte	20	70%
	qNSC	22	73%
	aNSC1	1	100%
	Vascular Endothelium	14	71%
miR-99a	aNSC1	4	25%
	aNSC2	2	100%
	Astrocyte	1	100%
	MFOL	2	50%
	Microglia	1	100%
	Neuron	2	50%
	qNSC	1	100%
	Vascular Endothelium	2	50%
miR-370	NSC	1	100%
miR-582	Astrocyte	1	0%
	aNSC1	1	100%
	Vascular Endothelium	1	100%
	Pericyte	1	100%
miR-1943	Neuron	1	0%
Average			72%

Table 2: Overlap of Tarbase validated target genes of miRNAs and expression of cell-type specific targets in single-cell SVZ data: miRNAs that have validated genes in TarBase are shown in column 1. Column 2 indicates which respective cell-types in the single-cell dataset that show differential expression of the pri-miRNA with age in the SVZ single-nucleus data set. Targets present in cell-type expression data indicates the number of target genes found to be expressed in the TarBase data and the single-cell dataset. The percentage of validated targets is the number of genes in column three that are changing in the appropriate direction given the pri-miRNA expression.

Declarations:

Ethics Approval:

Mouse tissues of strain C57BL/6 were collected in-house from male mice obtained from the NIA Aged Rodent Colonies (<https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook>). Mice were sacrificed in accordance with Brown University's IACUC guidelines (Protocol #19-01-0005).

Consent For Publication: Not Applicable

Availability of Data and Materials: Data is available, according to NIHGIMS practices. **The datasets generated during and/or analyzed during the current study will be publicly available upon publication.** 5XFAD and Wild-Type cortex dataset can be found at **GEO (GSE140511)** (18). The single-cell dataset of young and aged SVZ can be found at **BioProject PRJNA450425** (40).

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Authors' Contributions:

JK conceived the study. JK and AE designed the study. AE and BK conducted mouse husbandry and dissection. JK and AE conducted nuclei isolation and 10X Genomics library preparation. AE conducted all bioinformatic analyses while TN contributed to analysis of single-nucleus SVZ data. AE conducted qRT-PCR and analysis. JK and AE wrote the manuscript with input from authors.

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Figure 1: primiReference Design and Resulting Clustering of Cell Types in the Young and Aged SVZ

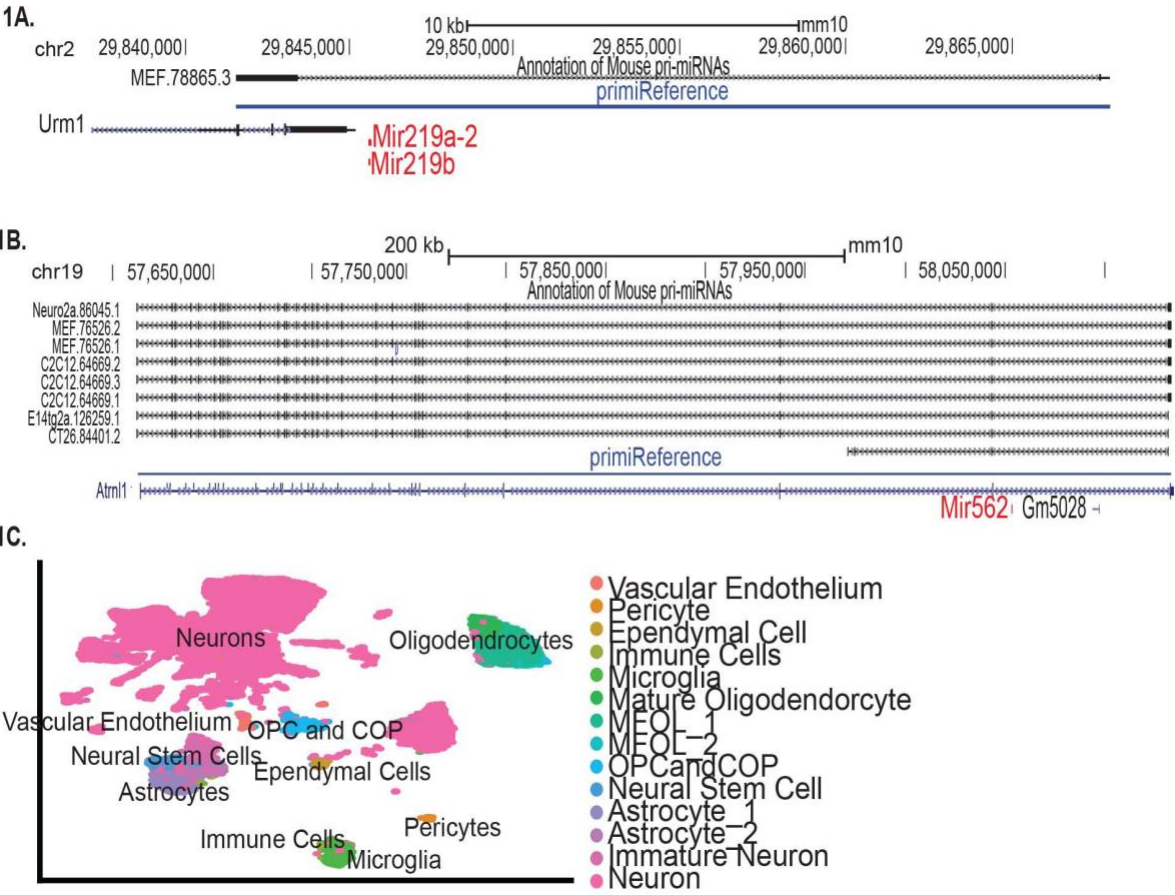


Figure 1: primiReference Design and Resulting Clustering of Cell Types in the Young and

Aged SVZ. Figure 1A: Depicted using the UCSC genome browser, the mouse embryonic fibroblast sequence of the primary miRNA for miR-219a-2 is shown. The primiReference generated alignment, shown in blue, is shown to overlap this intergenic primary miRNA sequence.

Figure 1B: Several cell lines from a publicly available dataset are shown to have pri-miRNAs mapping to the Atrn1 gene from which the primary-miR562 gene is transcribed intragenically as well. The primiReference alignment overlaps with the consensus of several cell lines.

Figure 1C: Fifty principal components were used to cluster cells in the young and aged SVZ, which is depicted by UMap dimensional reduction. The larger the global and local distances, the more distinct the cell types and nuclei are from one another.

Figure 2: Significantly Changing primary miRNAs in the Aged SVZ

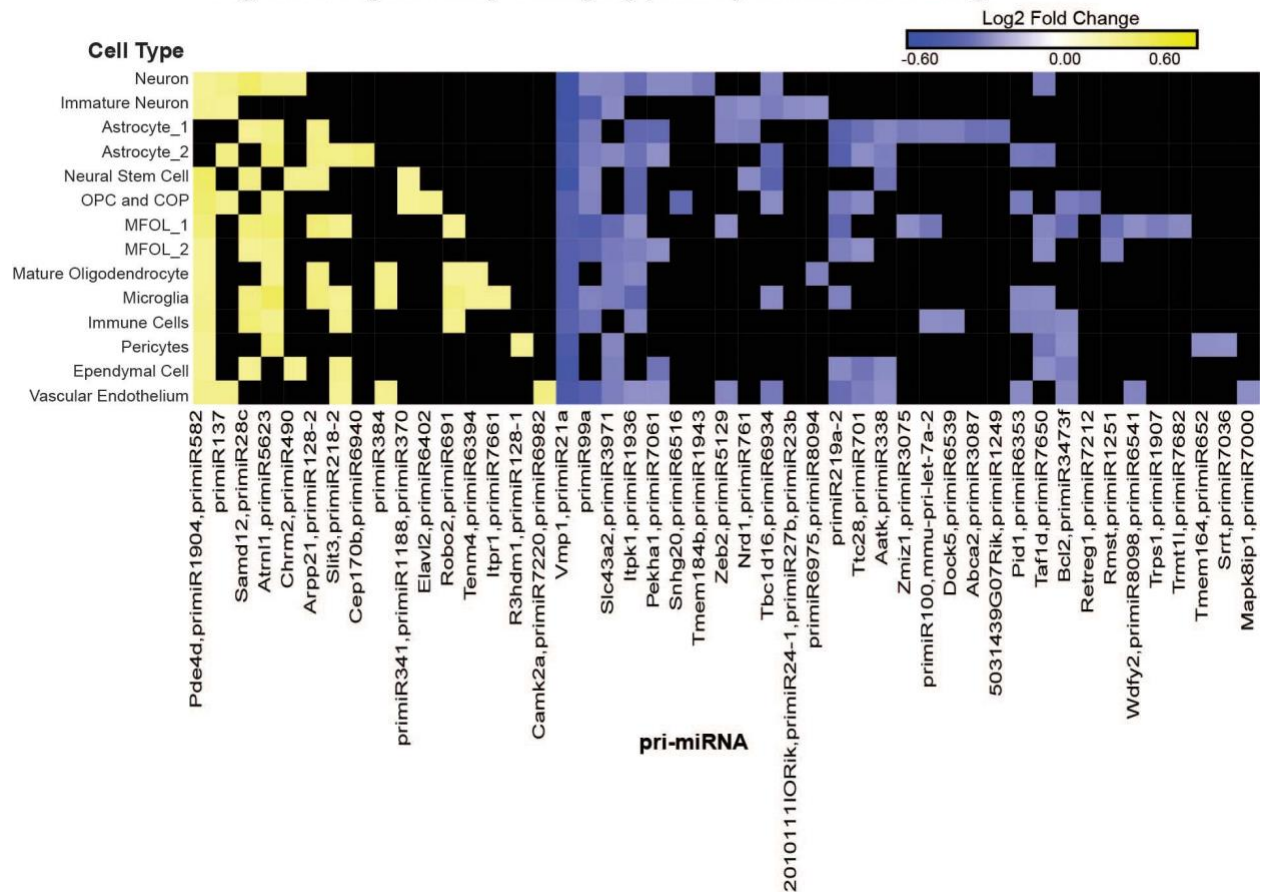


Figure 2: Significantly Changing primary miRNAs in the Aged SVZ. primary miRNAs with significant differences in expression between young and old SVZ ($p < .05$), per cell-type are shown by heatmap. Those increasing in expression with age are shown in yellow, with color depicted by the log fold-change while primary miRNAs decreasing in expression with age are shown in blue. Black cells represent cell-types that have no change in expression with age of a particular primary miRNA that other cell-types are shown to have changing significantly.

Figure 3: Validation of primiReference and Pathways of Predicted Target Genes of Differentially Expressed pri-miRNAs

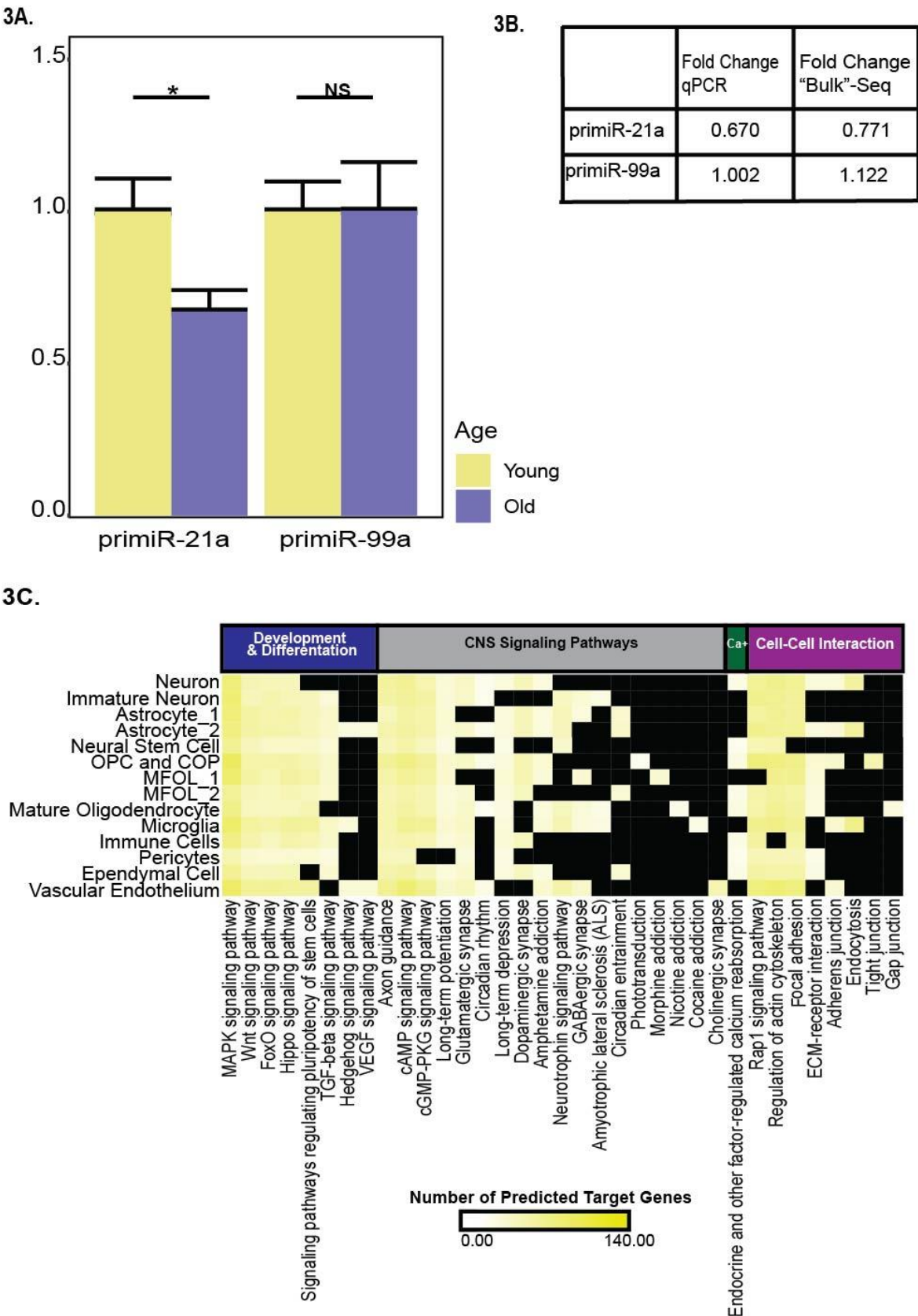


Figure 3: Validation of primiReference and Pathways of Predicted Target Genes of

Differentially Expressed pri-miRNAs. Figure 3A: The relative expression of primiR-21a and primiR-99a are shown from qPCR data. The yellow highlights the relative expression of whole young SVZ tissue, while the blue represents the relative expression of whole old SVZ tissue.

For primiR-21a the fold change between old and young SVZ was significant ($p < .05$). **Figure 3B:**

The table depicts the fold change values between old and young SVZ for primiR-21a and

primiR-99a in “Bulk” sequencing data and qPCR. **Figure 3C:** The pathways of predicted target

genes of differentially expressed pri-miRNAs, per cell-type, are depicted by the number of

genes predicted to be targeted per pathway. The KEGG pathways depicted in this heatmap

belong to the general categories of development and differentiation, CNS signaling pathways,

cell-cell interaction, and calcium signaling. All other pathways can be found in Supplementary

Table 4. Yellow is used to depict the number of predicted target genes enriched per KEGG

pathway of primary miRNAs changing with age

Figure 4: Clustering and pri-miRNA Differential Expression in WT and 5XFAD Cortex

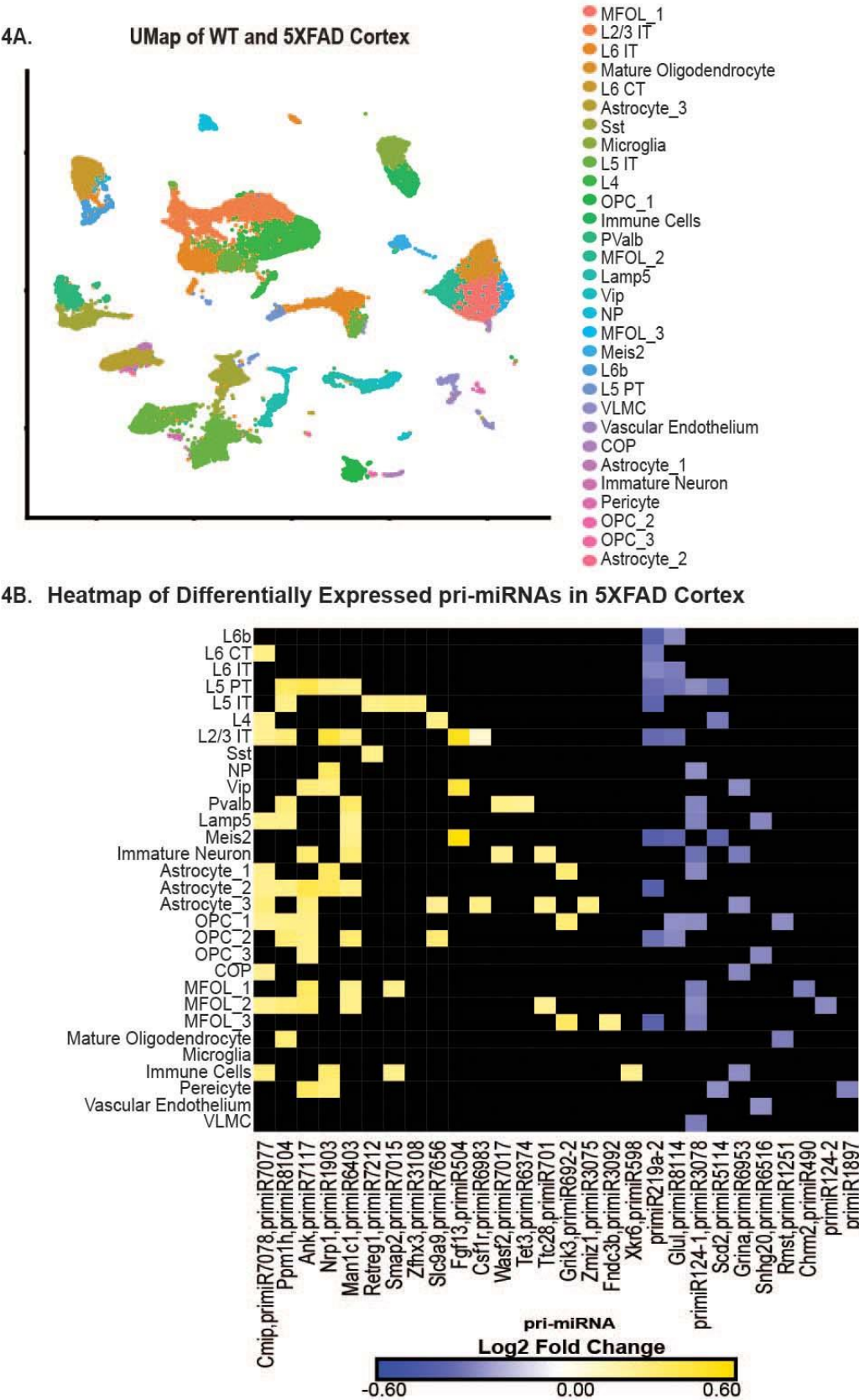


Figure 4: Clustering and pri-miRNA Differential Expression of Cell-Types in WT and

5XFAD Cortex: Figure 4A: Fifty principal components were used to cluster cells in WT and

5XFAD cortex, which is depicted by UMap dimensional reduction. The larger the global and

local distances, the more distinct the cell types and nuclei are from one another. **4B:** pri-

miRNAs with significant differences in expression between 5XFAD and wild-type cortex ($p < .05$),

per cell-type, are shown by heat-map. Those increasing in expression in 5XFAD compared to

wild-type are shown in yellow, with color depicted by the log fold change, while pri- miRNAs

decreasing in expression in the 5XFAD condition are shown in blue. Black cells represent cell-

types that have no change in expression between conditions of a particular pri-miRNA that other

cell-types are shown to have changing significantly.

Figure 5: Pathways of Predicted Target Genes of Differentially Expressed pri-miRNAs in the 5XFAD Cortex

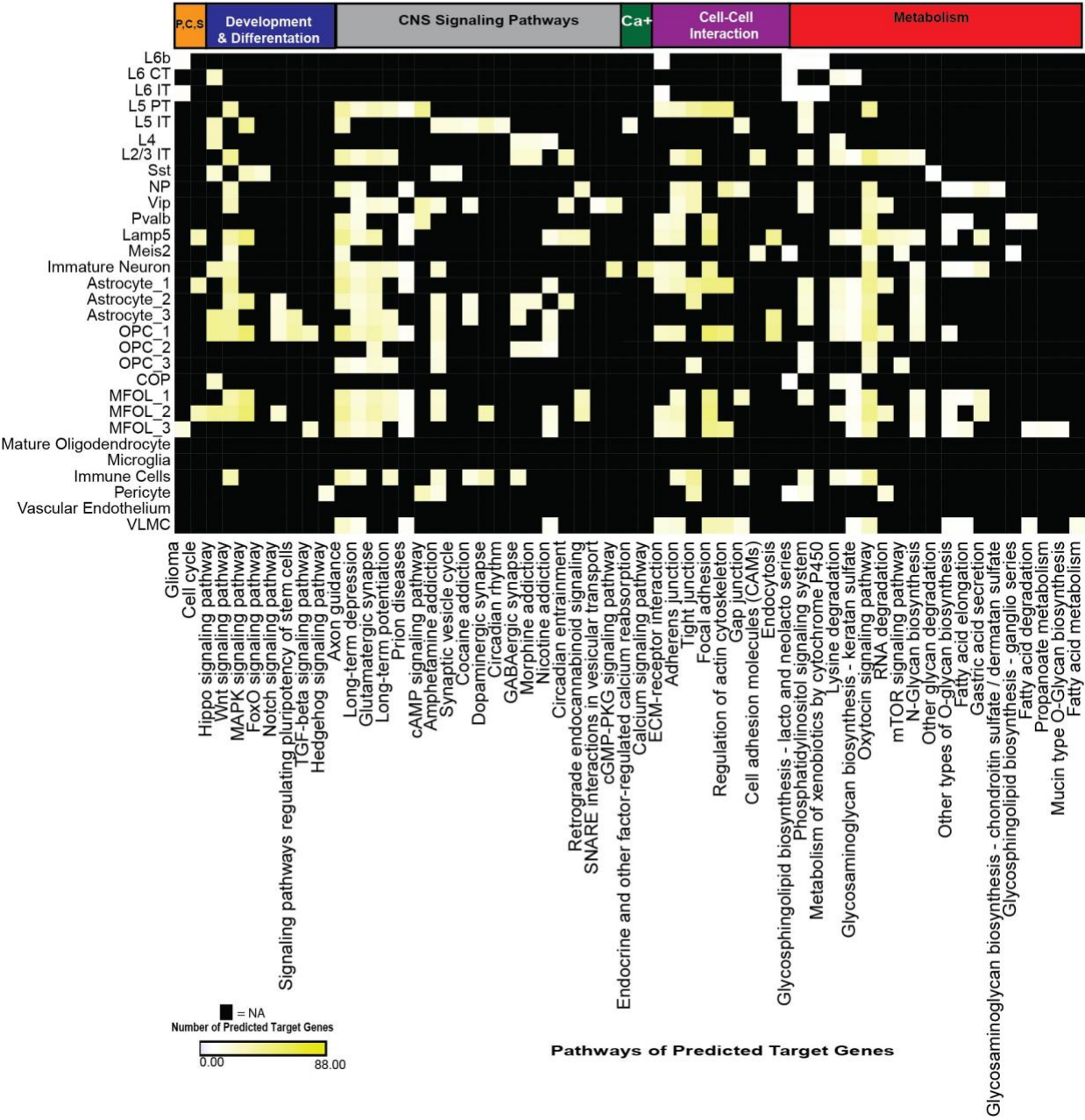
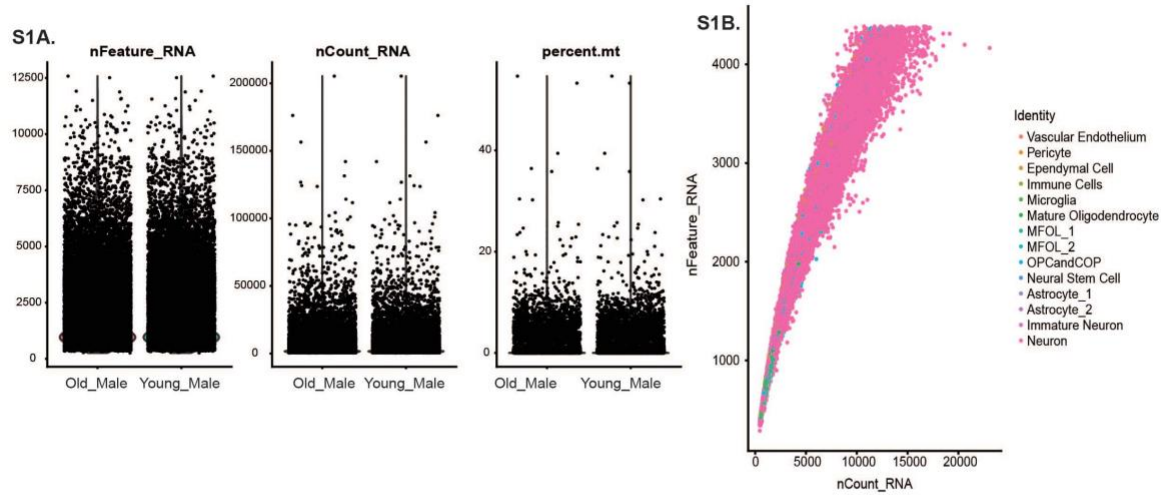


Figure 5: Pathways of Predicted Target Genes of Differentially Expressed pri-miRNAs in the 5XFAD Cortex: The mature sequences of primary miRNAs that are both increasing or decreasing in expression in the 5XFAD condition, per cell-type were used to computationally predict KEGG pathways enriched with predicted target genes. The KEGG pathways depicted in this heatmap belong to cell proliferation, cancer, and survival pathways (PCS) as well as pathways of development and differentiation, CNS pathways, calcium signaling, cell-cell interaction, and metabolism.

Additional File 1: Quality Control of Young and Aged SVZ Single-Nucleus Data



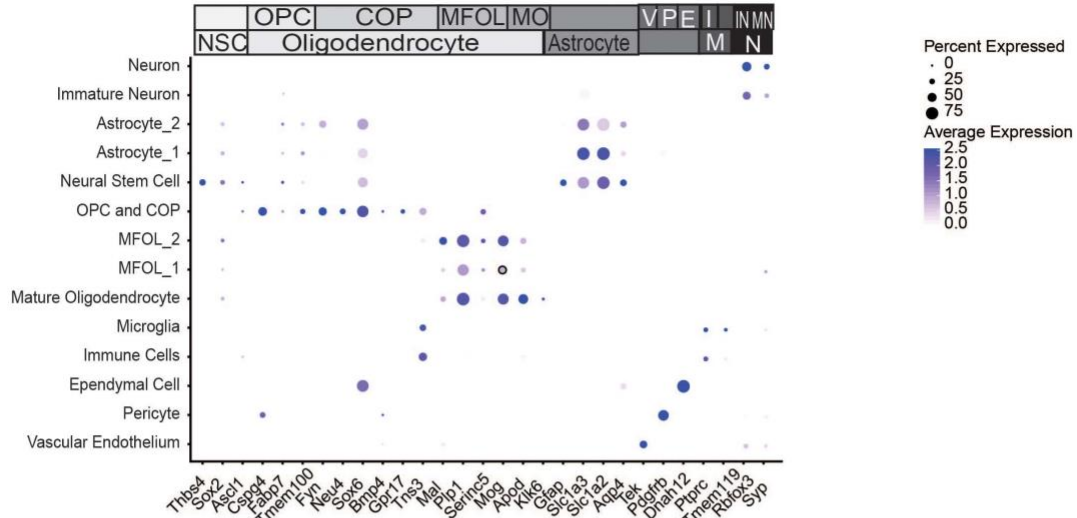
Additional File 1: Quality Control of Young and Aged SVZ Single-Nucleus Data

S1A: The number of features, counts, and percentage of mitochondrial content per cell in young and aged SVZ is shown via violin plot. Each dot is a cell and the cells shown represent pre-cell filtration settings. **S1B:** A feature scatter for the number of counts compared to the number of features is shown post-filtration to demonstrate a strong correlation of counts to features after quality control.

Supplementary Figure 2: Cluster Identities of Young and Aged SVZ and WT and 5XFAD Cortex

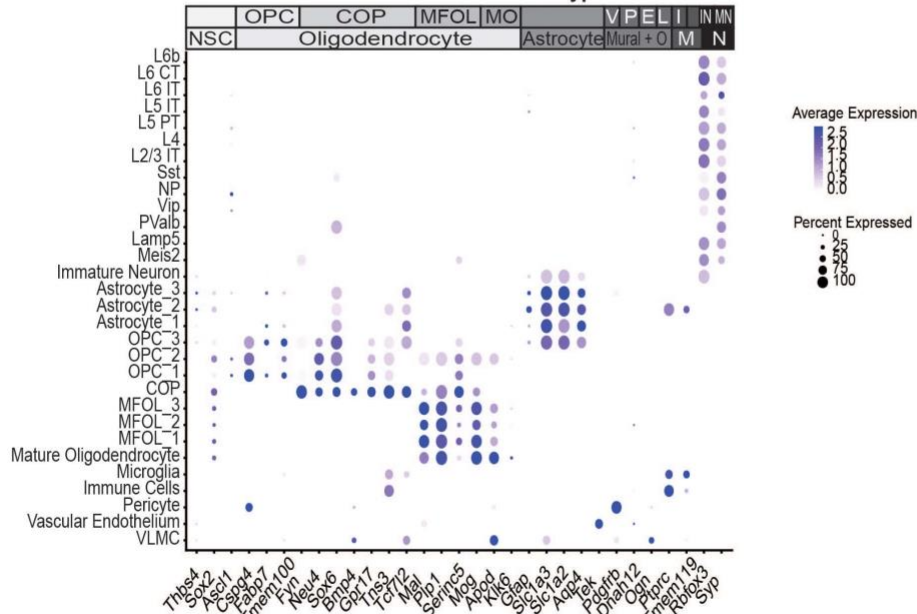
S2A

Cluster Identities of Young and Aged SVZ



S2B

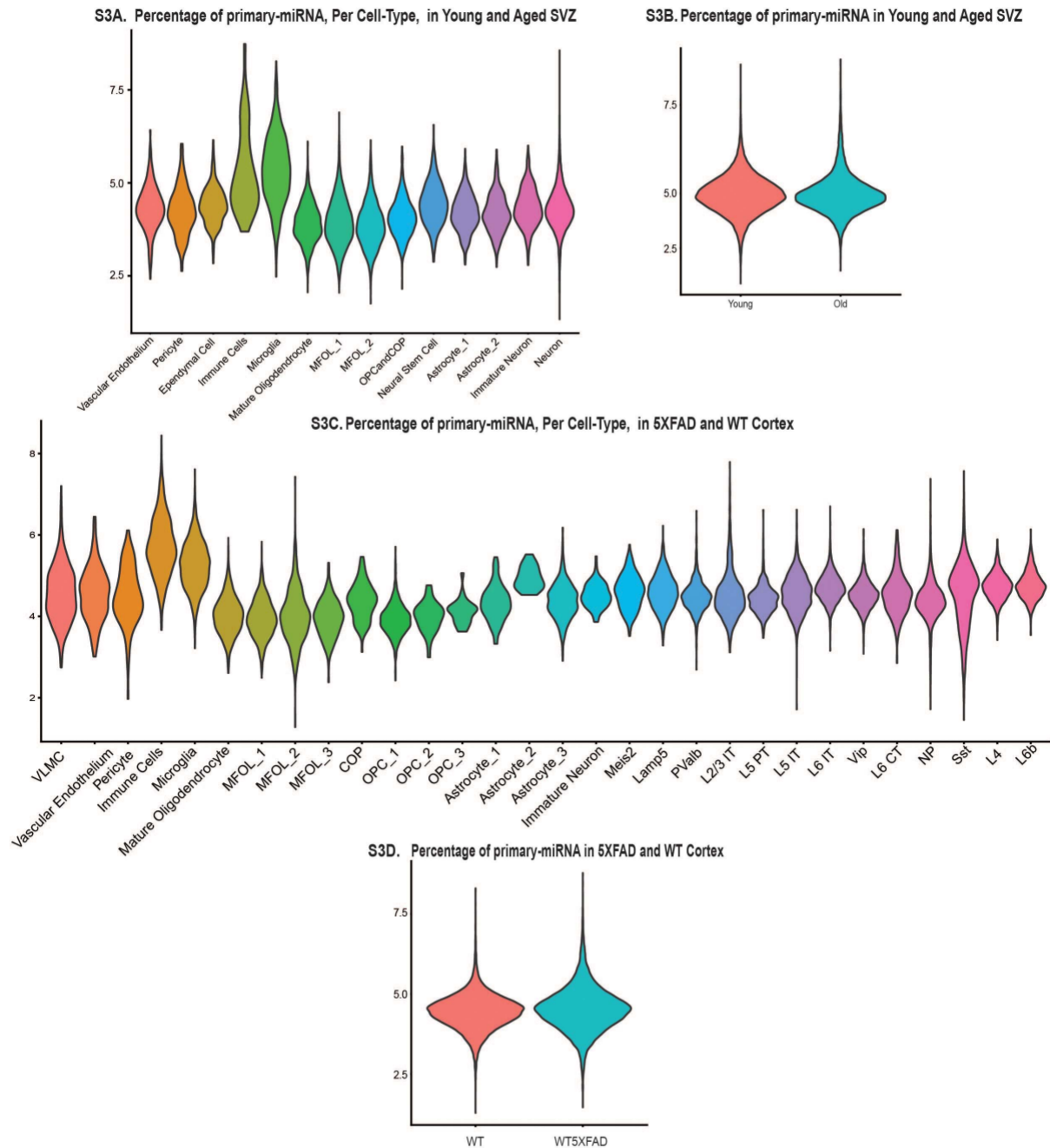
Cluster Identities of 5XFAD and Wild-Type Cortex



Supplementary Figure 2: Cluster Identities of Young and Aged SVZ and WT and 5XFAD Cortex.

S2A: Cluster Identities of Young and Aged SVZ A Dot Plot is used to depict cell-type specific expression of genes used to identify each cluster. The color is based on average expression per gene while the size of the dot depicts the percentage of cells within each cluster expressing the gene. Neural stem cells (NSCs) express *Thbs4*, *Sox2*, and *Ascl1* while oligodendrocyte progenitor cells (OPCs) express *Cspg4*, *Fabp7*, and *Tmem100* and committed oligodendrocyte progenitor cells (COPs) express *Fyn*, *Neu4*, *Sox6*, *Bmp4*, *Gpr17*, and *Tns3*. Myelin forming oligodendrocytes (MFOLs) express *Mal*, *Plp1*, *Serinc5*, and *Mog* while only newly formed oligodendrocytes express *Tcf12*. Mature oligodendrocytes express *Apod* and *Kik6*. Astrocytes express *Gfap*, *Slc1a3*, *Slc1a2*, and *Aqp4*. Vascular endothelium (V) express *Tek* while pericytes (P) express *Pdgfra* and ependymal cells express *Dnah12*. Cells of the choroid plexus (C) express *Ttr* while both microglia (M) and immune cells (I) express *Ptprc*, but only microglia express *Tmem119*. Mature neurons (MN) express *Syp* while immature neurons (IN) express *Rbfox3*. **S2B:** Cluster identities of 5XFAD and Wildtype clusters were defined as the SVZ above. Specific neuronal subtypes, however, were further defined using anchors from the Allen Brain Atlas.

Supplementary Figure 3: primary microRNA transcript abundance per cell in single-nucleus data



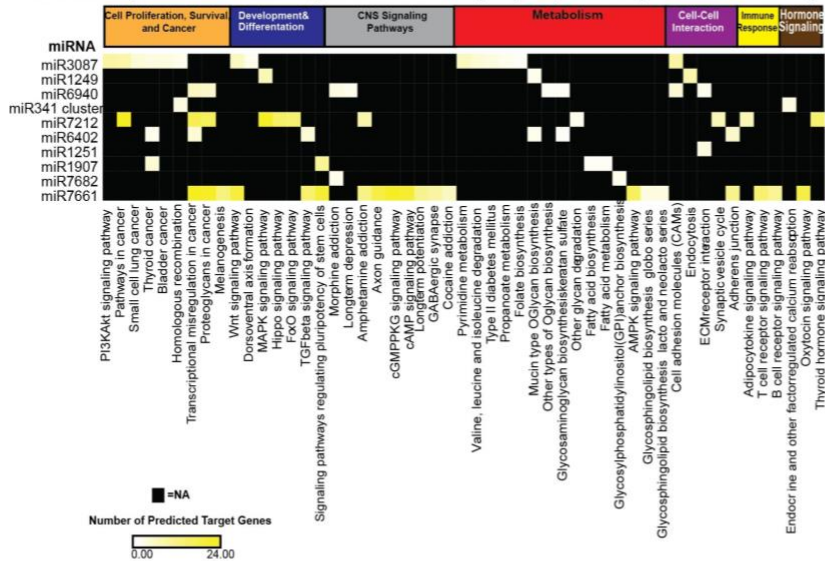
Supplementary Figure 3: primary microRNA transcript abundance per cell in single-nucleus data. **S3A:** Violin plots depicting primary microRNA transcript abundance per cell-type in each cluster identity demonstrate the amount of primary microRNA features as compared to total gene features in young and aged subventricular zone data. **S3B:** Violin plots depict primary microRNA transcript abundance per cell in each sample group, young and old subventricular zone, as a percentage of total feature abundance. **S3C:** Violin plots depicting primary microRNA transcript abundance per cell in each cluster identity demonstrate the amount of primary microRNA features as compared to total gene features in wild-type and wild-type 5XFAD cortex. **S3D:** Violin plots depict primary microRNA transcript abundance per cell in each sample group, wild-type and 5XFAD cortex, as a percentage of total feature abundance.

Supplementary Figure 4: Cell-Type Specific Expression of primary miRNAs with Age in the SVZ.

S4A. Table of Uniquely Expressed primary microRNAs per Cell-Type and Their Fold Change with Age in the Subventricular Zone

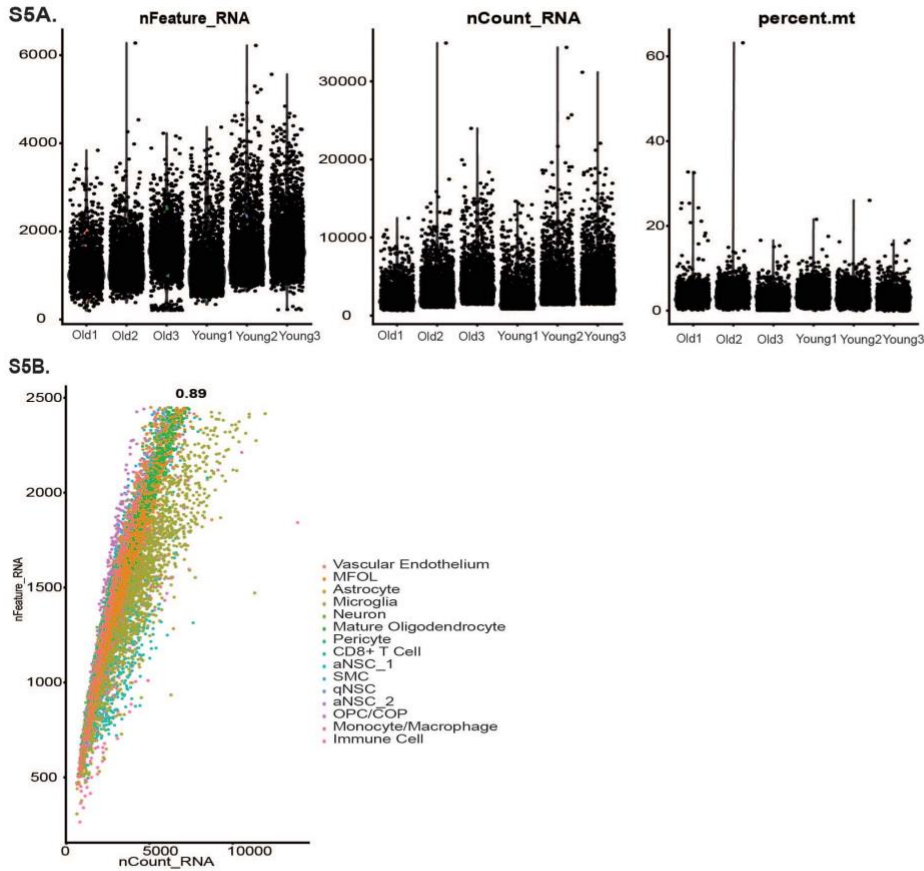
	Astrocyte_1	Astrocyte_2	Neural Stem Cell	OPCandCOP	MFOL_1	MFOL_2	Microglia
Abca2.primiR3087	-0.33						
5031439G07Rik.primiR1249	-0.32						
Cep170b.primiR6940		0.32					
primiR341cluster			0.25	0.26			
Retreg1.primiR7212				-0.32			
Elavl2.primiR6402				0.27			
Rmst.primiR1251					-0.33	-0.29	
Trps1.primiR1907					-0.30		
Trmt1l.primiR7682					-0.27		
Itpr1.primiR7661							0.26

S4B. Pathways of Predicted Target Genes of Uniquely Expressed pri-miRNAs Changing with Age in the Male SVZ



Supplementary Figure 4: Cell-Type Specific Expression of primary miRNAs with Age in the SVZ. **S4A:** Log fold-change of primary miRNAs found to be uniquely changing in specific cell-types are shown. **S4B:** The mature annotation of primary miRNAs uniquely changing in each cell-type were input into miRPath to determine enrichment of predicted target genes within KEGG pathways. The heatmap shows the number of predicted target genes within each pathway. These pathways fall into the categories of cell proliferation, survival, and cancer as well as development & differentiation, CNS signaling pathways, metabolism, cell-cell interaction, immune response, calcium signaling, and hormone signaling.

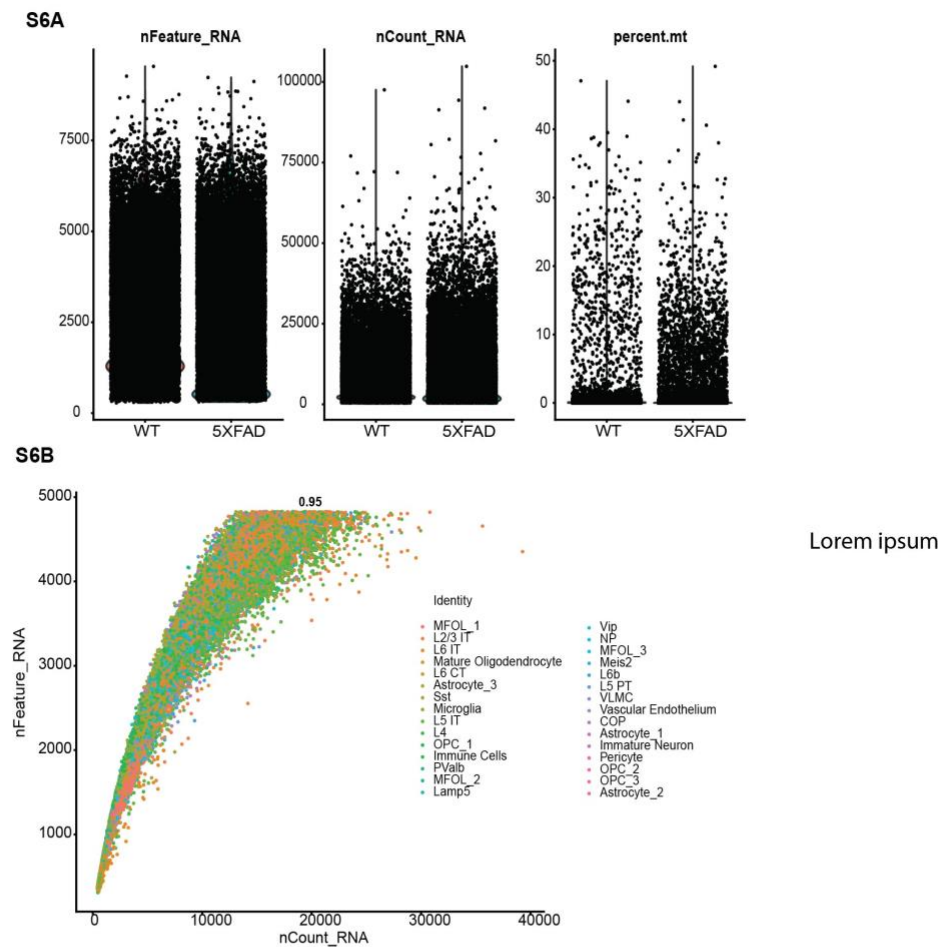
Supplementary Figure 5: Quality Control of Young and Aged SVZ Single-Cell Data



Supplementary Figure 5: Quality Control of Young and Aged SVZ Single-Cell Data

S5A: The number of features, counts, and percentage of mitochondrial content per cell in young and aged svz is shown via violin plot. The cells shown represent pre-cell filtration settings. **S5B:** A feature scatter for the number of features and number of counts of the features is shown to determine the relationship between counts and features per cell-type post-filtration.

Supplementary Figure 6: Quality Control of WT and 5XFAD Cortex Single-Nucleus Data



Supplementary Figure 6: Quality Control of WT and 5XFAD Cortex Single-Nucleus Data
S6A: The number of features, counts, and percentage of mitochondrial content per cell in WT and 5XFAD cortex is shown via violin plot. The data shown represent pre-filtration data. **S6B:** A scatter plot showing the number of features vs. the number of counts per nucleus post-filtration.

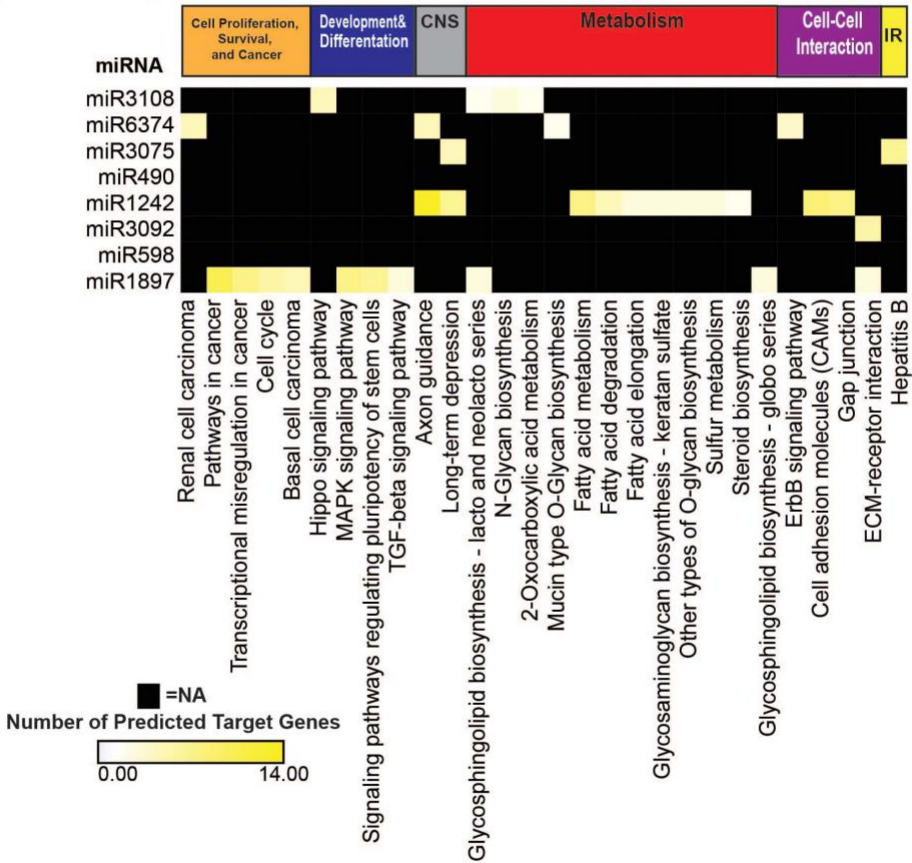
Supplementary Figure 7: Cell-Type Specific Expression of primary miRNAs in the 5XFAD Cortex compared to Wild-type

S7A Table of Uniquely Expressed primary microRNAs per Cell-Type and Their Fold Change in the 5XFAD Mouse Cortex

	L5 IT	Pvalb	Astrocyte_3	MFOL_1	MFOL_2	MFOL_3	Immune	Pericyte
Zfhx3,primiR3108	0.30							
Tet3,primiR6374		0.26						
Zmiz1,primiR3075			0.29					
Chrm2,primiR490				-0.29				
primiR124-2					-0.26			
Fndc3b,primiR3092						0.26		
Xkr6,primiR598							0.26	
primiR1897								-0.28

S7B

Pathways of Predicted Target Genes of Uniquely Expressed pri-miRNAs Changing in the 5XFAD Cortex Compared to Wild-Type



Supplementary Figure 7: Cell-Type Specific Expression of primary miRNAs in the 5XFAD Cortex compared to Wild-type S7A: Log fold-change of primary miRNAs found to be uniquely changing in specific cell-types are shown. **S7B:** the mature annotation of primary miRNAs uniquely changing in each cell-type were input into miRPath to determine enrichment of predicted target genes within KEGG pathways. The heatmap shows the number of predicted target genes within each pathway. These pathways fall into the categories of cell proliferation, survival, and cancer as well as development & differentiation, CNS signaling pathways, metabolism, cell-cell interaction, and immune response.

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Chapter 3:
Sex-Specific Differences in the Young and Aged Cell-Types of the SVZ

Abstract:

Background: Aging leads to decline of the subventricular (SVZ) niche of neural stem cells. The effect of sex on this aging process, however, remains elusive. Furthermore, the differences in gene expression between female and male individual cell-types within the SVZ is unknown in both young and old mice. Along with gene expression, differences in expression of regulatory RNAs, such as miRNAs, between the sexes remains unstudied. Therefore, the differences in pathway regulation between the sexes in both young and aged groups remains unknown for cell-types within the SVZ, and it is unknown if these differences lead to differential mechanisms of age-associated decline.

Results: In this study, we use primiReference to elucidate both gene and pri-miRNA expression in the young and aged SVZ cell-types of female and male mice. Age-associated transcriptional differences in Wnt signaling genes and calcium signaling genes were found between the sexes. When comparing young female and male cell-types, there were far more differences in gene and pri-miRNA expression while gene expression became more similar in a comparison of the aged cell-types. The pri-miRNA differences found between the sexes in the young cell-types are predicted to target pathways of development of differentiation while the pri-miRNA differences found between the sexes in the old cell-types are predicted to target more CNS signaling pathways.

Conclusions: With age, gene and pri-miRNA expression becomes more similar between female and male SVZ cell-types. On the other hand, there are key differences in transcriptional regulation of both the Wnt and calcium signaling pathways between female and male cell-types that will need to be further investigated.

Background

In the aging subventricular zone of the brain (**SVZ**), neural stem cell (**NSC**) number, self-renewal capacity, and neurogenic potential declines (1-3). In the young SVZ, NSCs differentiate into neurons, oligodendrocytes, and astrocytes during asymmetric division giving rise to a progenitor cell and self-renewed NSC (4-7). During neurogenesis, NSCs give rise to transiently amplifying cells (TAPs) which can divide and differentiate into neuroblasts, which then migrate through the rostral migratory stream towards the olfactory bulb. Once in the olfactory bulb, neuroblasts differentiate into interneurons through various signaling mechanism (8-13). Meanwhile, during oligodendrogenesis, these progenitors take on oligodendrocyte progenitor (OPC) characteristics and give rise to committed oligodendrocyte progenitors and then oligodendrocytes (14-16). In the aging brain, however, the cell-cell and intracellular signaling mechanisms guiding NSC pool maintenance, neurogenesis, oligodendrogenesis, and astrogenesis become dysfunctional (1, 3, 17-25).

Although the study of age-associated changes in the SVZ has been popular area of study, the majority of studies using animal models, to date, have been conducted using males (26, 27). In both human and mouse, there have been studies documenting the wide array of differences sex-hormones play in the aging process thus leading to sexually dimorphic aging (27-29). With respect to studies on sex-steroids and neurogenesis, *in vitro* studies have found that estrogen exposure can increase neurogenesis if given in 10 nM concentrations. The amount of estrogen given and the age of the NSCs (embryonic versus adult) are additional variables that have caused controversy in the field over whether estrogen truly leads to increased neurogenesis (30, 31). Furthermore, growth factors in the media such as Fgf2 and EGF resulted in difference in preference for neurogenic versus oligodendrocyte lineage, with Fgf2 exposure leading to a more oligodendrocyte lineage (31-33). By using *in vitro* studies, however, cell-signaling mechanisms regulating NSCs, and the effects of sex-steroids on these

other cell-types are neglected as factors in determining NSC pool maintenance and differentiation.

At baseline, *in vivo*, female mice have a higher proportion of NSCs and a higher rate of proliferation in the SVZ than in males (34, 35). Interestingly, estrogen *in vivo* causes a decrease in BrdU incorporation while castration of male mice, limiting testosterone, increases BrdU incorporation (36). For both *in vivo* and *in vitro* studies on sexual dimorphism in neurogenesis, there has been few studies looking at sexual dimorphism on aging in the SVZ. Using Long-Evans rats, Waldron et al. isolated NSCs from 20-month-old and 3-month-old SVZ. The NSCs were then differentiated using retinoic acid, and the cell-types for which they differentiated into were quantified. This study uncovered a preference for astrogenesis among female mice, and a reduction in neuroblast number for both male and female rats, including a further decrease in the female rats (26). This study underscores the need for more profiling of sexual dimorphism in the aging SVZ.

For these differences in NSC maintenance and differentiation to occur, with age, in a sex-specific manner, there must be differences in gene expression and regulation between the sexes and ages. In the studies conducted to date, no study has profiled the differences in gene expression and regulation of SVZ cell-types in both young and old female and male mice which may provide mechanisms for these differences. Since cell-cell signaling is vital to NSC differentiation, it is important to determine if there are changes in gene expression of these pathways that are sexually dimorphic with age (11, 12, 37-42). To date, several miRNAs have been described to regulate pathways involved in neurogenesis, and their impact on NSC maintenance and differentiation is significant (43-49). On the other hand, none have been characterized by sex and age within each cell-type.

Therefore, in this study, we described the pri-miRNA and gene expression profiles of young and aged female and male SVZ in a cell-type specific manner using single-nucleus sequencing. From these profiles and subsequent pathway analyses, female and male mice

have far greater differences in gene and pri-miRNA expression in the young SVZ cell-types. In the old SVZ cell-types, gene and pri-miRNA expression becomes more similar. There are some genes, however, that change in opposite directions between female and male mice with age in the SVZ that may either provide benefit or disadvantage to the aging process in the SVZ in a sex-specific manner. Further studies will be needed to both validate and interrogate these genes and processes to determine the full impact of sex-specific gene and pri-miRNA expression on SVZ health.

Results

Neural Stem Cells and Their Progenitors Decline in Number with Age in Both Sexes

To discern the age-associated changes in cell-type specific gene and pri-miRNA expression in male and female SVZ, single-nucleus sequencing was conducted. This dataset consists of four samples from each group (i.e., young female, young male, old female, old male). Following sequencing, alignment, and quality control filtration, 16 clusters were identified (**Figure 1A**). Neuronal subclusters were not separated, and neurons were grouped into one large class since these were likely striatal border contamination since the SVZ does not have distinct borders. The neurons were thus minimally analyzed in this study. Conversely, the more abundant cell types of the SVZ were additionally identified such as ependymal cells, astrocytes, NSCs, TAPs, neuroblasts, OPCs, COPs, myelin forming oligodendrocytes (MFOLs), mature oligodendrocytes, microglia, pericytes, and vascular endothelium (**Figure 1**).

Interestingly, with the abundance of nuclei, principal component analysis and subsequent UMap reduction was able to clearly separate clusters of NSCs from their progenitor cell lineage such as TAPs, neuroblasts, OPCs, and COPs (**Figure 1**). Furthermore, the global distance is quite small between the TAP, neuroblast, and immature neuron clusters showing distinct yet shared expression of many genes since they are all from the same lineage (**Figure 1**). As other studies have noted, the number of NSCs and their progenitors declines with age

(2, 3, 18). Likewise, the number of NSCs and their progenitors has sharply declined in this dataset with age in both females and males (**Supplementary Table 1**). NSC number is reduced further in the males than in the females with age, intriguingly.

Age-Associated Changes in Gene Expression Within the Female and Male SVZ

Age-Associated Changes in the Cell-types of the Female SVZ

To determine if cell-types in the female and male SVZ show similar patterns in age-associated gene expression, differential expression was conducted between each cell-type comparing the old to the young within each sex (**Supplementary Table 2, Supplementary Table 3**). Interestingly, *Tsix*, the antagonist to *Xist*, *expression* is increased in every cell-type with age. *Tsix* is transcribed antisense to *Xist* and leads to differential chromatin organization (50-52). Therefore, the effects of increased *Tsix* expression within every cell-type should be further investigated to determine if this leads to increased or decreased expression of X chromosome genes with age.

To elucidate the cell-type specific impact of age-associated changes in gene-expression on pathways, ClusterProfiler was employed to determine which differentially expressed genes were overrepresented in KEGG pathways (**Supplementary Table 5**) (53). Unlike gene set enrichment analysis, overrepresentation analysis does not factor in the direction of change for differentially expressed genes. This is beneficial when looking at pathways that include increases in inhibitors of pathway transcription, for example. NSCs and their progenitors have, overall, similar pathways overrepresented by changes in age-associated differential expression (**Figure 2B, Supplementary Table 5**). All five cell-types shown, in addition to the surrounding cell-types such as astrocytes, ependymal cells, microglia, and vascular endothelium, have overrepresentation of differentially expressed genes involved in pathways of neurodegeneration – multiple diseases (**Figure 2B, Figure S1**). NSCs, Neuroblasts, OPCs, and COPs have more

overrepresentation in the more specific neurodegenerative pathways of Parkinson's Disease and Huntington's Disease (**Figure 2B**). In addition, astrocytes have overrepresentation of differentially expressed genes in the Alzheimer's Disease and Prion Disease pathways (**Figure S1**). It is paramount to further interrogate the genes found to be overrepresented in these pathways with age since females are disproportionately affected by neurodegenerative disease (54).

In addition, there was also an overrepresentation of differentially expressed genes in the estrogen signaling pathway in all of these cell-types except TAPs (**Figure 2B**). Many of these genes such as *Calm1* and *Adcy1* are involved in calcium signaling, and they were upregulated with age. On the other hand, *Creb*, which can regulate expression of genes via induction of a signaling cascade leading to binding of transcription factors to an estrogen response element (ER) when in the presence of estrogen, is decreased in expression with age (**Supplementary Table 5**) (55).

Although similar overrepresentation of differentially expressed genes for all cell-types was shown, it is worth noting some key differences. TAP and OPC cell-types have significant overrepresentation of differentially expressed genes in the calcium signaling pathway. Although many of the pathways overrepresented contain genes also involved in calcium signaling, such as many pathways of secretion and different types of synapses, these two cell types still have far greater overrepresentation. Furthermore, the NSC, TAP, and OPC clusters have overrepresentation within the insulin signaling pathway, which is interesting since insulin signaling regulates processes like FoxO signaling (**Figure 2B**).

Age-Associated Changes in the Cell-types of the Male SVZ

There were far more downregulated genes in the male cell-types with age than in the female cell-types (**Figure 2A, Figure 3A**). Overall, 180 genes were found to be differentially expressed in every cell-type (**Supplementary Table 3**). From these 180 genes, two

transcription factors emerged as regulators of gene expression via Ingenuity Pathway Analysis (Qiagen IPA). SOX2 and Tcf7l2 were found to be inhibited (z score < -2) based on the expression of their targets within our data (56). Interestingly, 36 of 37 genes found to be regulated by Tcf7l2 are found to decrease in expression across cell-types (**Supplementary Tables 3 & 4**). Tcf7l2 is a transcription factor involved in maintaining insulin response and Wnt/ β -catenin signaling (57-59). Therefore, it will be interesting in future studies investigate the effects of decreased expression of many Tcf7l2 targets on NSC differentiation due to the involvement of these genes in these pathways.

Analogous to female cell-types, the cell-types of the male SVZ had similar overrepresentation of differentially expressed genes in pathways. Unlike female cell-types, however, the pathway with the greatest overrepresentation, based on the number of differentially expressed genes, is the MAPK signaling pathway (**Figure 3B, Figure S2, Supplementary Table 6**). This pathway, however, is only overrepresented in the NSC, TAP, astrocyte, microglia, and ependymal cell types (**Figure 3B, Figure S2**). Even more so than the female cell-types of the SVZ, the NSCs and their progenitors have very similar, overlapping pathways of overrepresentation. Many of these differentially expressed genes belonging to different pathways are involved in calcium signaling. NSC, TAP, and astrocyte clusters have direct overrepresentation of the calcium signaling pathway (**Figure 3B, Figure S2, Supplementary Table 6**). This pathway does not include every calcium signaling gene, however, and there is overlap of many of these genes with smaller pathways, which leads to more overrepresentation of more obscure pathways like taste transduction. Together, this data shows that calcium signaling genes are differentially expressed with age in the cell-types of the male SVZ, and they are involved in regulating several pathways.

Differences in Gene Expression and Overrepresentation Analysis Between the Sexes with Age

Since many genes involved in calcium signaling led to overrepresentation of particular pathways in both female and male cell-types with age, it is important to disentangle how many differentially expressed genes are overlapping between these comparisons. Interestingly, the majority of genes differentially expressed with age are unique to either sex in NSCs, TAPs, and Neuroblasts (**Figures 4A-4C**). Comparing the pathways of overrepresentation with age between female and male NSCs, it becomes more obvious that, although many there are many shared pathways, the actual genes involved are likely different (**Figure 4D**). For example, pathways where both female and male NSCs have overrepresentation, the overrepresentation is more statistically significant and the number of genes overrepresented is greater in the male group (**Figure 4D**). In addition, only the top 20 pathways are represented in this figure. Other pathways such as Wnt and Notch are overrepresented by genes changing in expression with age in the male NSCs, while these pathways are not overrepresented in the female NSCs with age (**Supplementary Table 6**).

Differences in Wnt Signaling with Age in Female and Male Cell-Types

Although Wnt signaling is overrepresented in male NSCs with age and not female NSCs, female NSCs have a statistically significant transcriptional decrease in the expression of *Tcf7l2* with age. This decrease is additionally observed in other cell-types within the female SVZ (**Supplementary Table 2**). Although the cell-types of the male SVZ have predicted inhibition of *Tcf7l2*, no such transcriptional decrease is observed with age. On the other hand, male cell-types have a decrease in *Daam1* expression with age (**Supplementary Table 3**). *Daam1* activates Wnt signaling by interacting with *Dvl* and *Rho* (60). It will be important for future studies to detangle whether differential transcriptional control of these two genes contributes to differential Wnt signaling with age in the SVZ.

Age-Associated Changes in Calcium Signaling

When extricating the actual genes differentially expressed within the overrepresentation analysis, the same genes kept appearing in multiple pathways. *Cacna1c* and *Calm1*, along with other calcium signaling associated genes, appeared in most pathways and cell-types. *Cacna1c* decreases in expression with age in females and increases in expression in males with age while *Calm1* increases in expression in both sexes with age (**Figure 4E and Figure 4F**). *Cacna1c* is a subunit of the L-type voltage-gated calcium ion channel CaV 1.2, and *Calm1* regulates CaV1.2 activity (61, 62). Interestingly, *Cacna1c* has an estrogen response element for transcription (63). This finding led us to explore deeper into other calcium signaling transcripts differentially expressed with age.

In NSCs, *Cacna1c* is unchanged with age in females, and it appears that most genes that are differentially expressed in the calcium signaling pathway are increased in expression in the female and male NSCs with age (**Figure 5A and Figure 5B**). On the other hand, in TAPs, another story unfolds. In female TAPs, both *ErbB4* and *Cacna1c* are downregulated with age, so calcium entry may be reduced. Interestingly, *ErbB4* also has an estrogen response element (64). Downstream calcium signaling genes, however, are increased in expression with age in females (**Figure S3A**). In the male TAPs, on the other hand, calcium channels *Cacna1c*, *Cacna1a*, *Chrm3*, and *Grin1* are increased in expression with age as well as some of their downstream effectors (**Figure S3B**). Together, this data indicates calcium signaling is different within different cell-types with age. Additionally, calcium signaling may be differentially regulated, depending on the ultimate result of signal transduction due to differential transcriptional regulation of calcium channels, between female and male cell-types with age in the SVZ.

Sex-Specific Changes in Gene Expression with Age in the SVZ

Since there were key differences unearthed in aging of SVZ cell-types between the sexes, it was necessary to understand whether these cell-types are more similar in expression

between young groups, aged groups, or were completely dissimilar in both age groups between the sexes. Therefore, age-matched comparisons were conducted by comparing female cell-types to male cell-types. These comparisons will be referred to as young female compared to young male (YF v YM) and old female compared to old male (OF v OM) for simplicity. The comparison of YF v YM yielded more differential gene expression than OF v OM (**Figure 6A**). When overlapping this gene expression between the age-matched comparisons, far more genes were uniquely differentially expressed in the young sex-specific comparison than in the old comparison (**Figure 6A-D**). In NSCs, 120 genes were differentially expressed only between YF v YM while only 63 genes were just expressed in the OF v OM comparison (**Figure 6B**). For NSCs and TAPs, there were 35 genes and 39 genes in common between the age-matched sex-specific comparisons, respectively (**Figure 6B and 6C**). In neuroblasts, even fewer genes are in common between the comparisons, with only 18 genes being differentially expressed in both YF v YM and OF v OM comparisons (**Figure 6D**). Some of the genes in common, however, were genes specific to one sex or another, such as genes on the Y chromosome or genes involved in X inactivation (**Figure 6A, Supplementary Table 7, and Supplementary Table 8**).

Although gene expression is more similar in the OF v OM comparison than in the YF v YM comparison, the pathways overrepresented by differentially expressed genes are mostly intersecting (**Figure 6E, Supplementary Table 9, and Supplementary Table 10**). The TAP cell-type was chosen to display differences in overrepresentation analysis since the TAPs were the only cell-type in which 20 or more pathways were overrepresented in both the YF v YM and OF v OM comparisons. Additionally, in the OF v OM comparison some cell-types did not meet the requirement for overrepresentation analysis since fewer genes were differentially expressed. The cell-types absent of overrepresented pathways for differentially expressed genes between the old female and old male SVZ are NSCs, OPCs, microglia, and vascular endothelium. In addition, it is known that TAPs decrease in number with age due to decreased NSC

proliferation, but it is additionally interesting to explore if there are transcriptional differences which may keep their number low (3).

In the TAPs, as well as other cell-types with fewer overrepresented pathways, the common pathways have more statistical significance and more differentially expressed genes represented within them for the YF v YM comparison (**Figure 6E, Supplementary Table 9, Supplementary Table 10**). Unique to the YF v YM comparison, the estrogen signaling pathway is overrepresented in the female TAPs compared to the male TAPs. This is intuitive since estrogen levels are much higher in young female mice than young male mice (65). Furthermore, there is overrepresentation of insulin secretion due to differences in gene expression between young females and males. It has been reported that sex-steroids have a different effect on this pathway, with testosterone leading to increased resistance. The Alzheimer's Disease pathway, on the contrary, was overrepresented by the differentially expressed genes in the old female brain compared to the male brain but not in the young.

Sex-Specific Differences in Calcium Signaling with Age

The pathways in common between YF v YM and OF v OM comparisons are mostly linked by differential expression of calcium signaling genes, although the calcium signaling pathway itself did not meet criteria for overrepresentation. In the YF v YM comparison, young female TAPs had increased expression of *Cacna1c*, *Chrm2*, and *Chrm3* compared to male TAPs (**Figure 7A**). *Chrm2* and *Chrm3* are cholinergic muscarinic receptors. Although little has been studied as to their exact role in SVZ neurogenesis, it is speculated that they contribute to signaling guiding proliferation (66). *Adcy1*, *Calm1* and *Gnas*, however, are decreased in expression in young females compared to young males (**Figure 7A**). *Adcy1*, type 1 adenylyl cyclase, is a brain-specific enzyme used to catalyze cAMP production (67) while *Gnas* is a subunit of the protein $G\alpha$ which activates some adenylyl cyclase proteins (68). *Calm1*, calmodulin 1, is a calcium sensor.

In the old comparison, however, *Cacna1c* is decreased in expression in female TAPs compared to male TAPs, and *Calm1* is increased in expression in the female TAPs compared to the male TAPs (**Figure 7B**). *Gnas*, *Adcy1*, *Chrm2*, and *Chrm3* expression are unchanged in the old comparison. *Chrm2* and *Chrm3* agonists are affected by estrogen, so decreased estrogen may play a role in the similarity of gene expression in the aged comparison (69, 70). Jointly, this data demonstrates a switch in the transcriptional profiles of calcium signaling in a sex and age dependent manner. Although many of these genes have not been investigated in the context of neurogenesis, the need for further investigation is warranted from this data.

Sex-Specific Differences in pri-miRNA Expression Within the Young and Aged SVZ

To fully grasp the sex-specific changes in gene-expression in young and aged SVZ cell-types, pri-miRNA expression was investigated. Since miRNAs have a significant impact on gene-expression it is imperative to elucidate if there is sex-specific differential expression of these pri-miRNAs in specific cell-types and if this expression changes between the sexes with age. To this end, *primiReference*, described in chapter 2, was used to align the sequencing files of this dataset to the genome, so pri-miRNA expression was available for interpretation. Subsequently, the impact of differentially expressed pri-miRNAs was examined via use of *miRPath* to determine if particular pathways are enriched for target genes of the mature form of these pri-miRNAs (71). By doing so, both gene and small RNA impact on sex-driven aging was assessed in this study.

Differential Expression of pri-miRNAs Between Young Female and Young Male SVZ Cell-Types May Impact Development and Differentiation

When comparing pri-miRNA differential expression between young female cell-types and their respective young male cell-types, many of the age-associated changes in pri-miRNAs referenced in chapter 2 are found. Expression of the polycistronic pri-miR-1904 and pri-miR-

582 transcript is increased in various young female cells compared to young male cells, and it is additionally increased in an age-associated manner as shown in Chapter 2, for example (**Figure 8A**). In the stem and progenitor cell-types, pri-miR-128-1 and pri-miR-128-2 are increased in expression in the female compared to the male. Interestingly, miR-128-1 targets IGF-1, while miR-128-2 targets stem cell factors such as KLF4 (**Table 1**) (48, 72). Female TAPs, neuroblasts, and OPCs have increased expression of pri-miR-490 compared to their respective male cell-types. pri-miR-490 represses HDAC2, which is implicated in the switch to committed progenitor (73, 74) (**Table 1 and Figure 8A**). Taken together, there may be some differences in NSC regulation and differentiation between young females and males, although the outcome of these differences is indiscernible without future studies.

For astrocytes, neuroblasts, OPCs, mature oligodendrocytes, and vascular endothelium, there is increased expression of the polycistronic transcript pri-miR-1904 and pri-miR-582 in females compared to their respective male cell-types. miR-582 is involved in regulating genes involved in the apoptosis pathway and inflammation (75). Interestingly, NSCs and TAPs have few pri-miRNAs that increase in expression between the sexes. Meanwhile, neurons have the most pri-miRNAs increasing in expression in females compared to males (**Figure 8A**). Unfortunately, most of these pri-miRNAs do not have known targets published in literature. Likewise, several pri-miRNAs are decreased in expression in the female cell-types compared to the male cell-types do not have any known targets in literature. Of note, the polycistronic transcript pri-miR-7220 and pri-miR-6982 is decreased in every cell-type in the female SVZ compared to the male SVZ. Interestingly, these miRNAs are transcribed as introns in the Camk2a gene, which is involved in calcium signaling (**Figure 8A**).

Since many of these pri-miRNAs do not have published targets in literature, miRPath was employed to determine the computationally predicted targets of these pri-miRNAs and their enrichment in particular pathways (**Figure 8B, Supplementary Table 11**). Astrocytes, TAPs, and vascular endothelium have pathway enrichment for predicted target genes of pri-miRNAs

differentially expressed, between female and male cell-types, in pathways involved in development and differentiation. All three cell-types have pri-miRNAs that are differentially expressed between the females and males that are involved in signaling regulating pluripotency of stem cells and Wnt signaling. Remarkably, differentially expressed pri-miRNAs, between the sexes, are predicted to target the Wnt pathway were found in MFOLs, NSCs, TAPs, neuroblasts, OPCs, and astrocytes (**Figure 8B, Supplementary Table 11**). NSCs, TAPs, Neuroblasts, and OPC cell-types are strongly regulated by Wnt signaling. In addition to this finding, astrocytes have the most pathways falling under the cell-cell interaction category for predicted targets of differentially expressed pri-miRNAs between the sexes (**Figure 8B, Supplementary Table 11**). This finding is key since astrocyte interactions drive neurogenesis.

Few pri-miRNAs are Differentially Expressed Between Old Female and Old Male SVZ Cell-Types

Unlike the comparison of young female SVZ to young male SVZ, the comparison of old female SVZ to old male SVZ yielded few cell-type specific differences and few differences overall in pri-miRNA expression. Every female cell-type has an increase in expression of the polycistronic transcript pri-miR-7220 and pri-miR-6982, which are contained within introns in the Camk2a gene, compared to their respective male cell-type (**Figure 9A**). In the young SVZ, however, the opposite was shown, with this transcript decreasing in expression in the female compared to the male (**Figure 9A**). Further investigation will be needed to determine the targets of these pri-miRNAs and the effects of this change in expression between the sexes with age. Like the change shown for the polycistronic transcript pri-miR-7220 and pri-miR-6982, pri-miR-137 was increased in expression in the female astrocytes compared to the male astrocytes in the young SVZ, but pri-miR-137 is decreased in expression between the female astrocytes and male astrocytes in the old SVZ (**Figure 9A**). This is fascinating since pri-miR-137 may have protective effects against inflammation (76) (**Table 1**). Conversely, pri-miR-5114 is

decreased in expression in young female NSCs compared to young male NSCs, but pri-miR-5114 is increased in expression in old female NSCs compared to old male NSCs (**Figure 8A and Figure 9A**). pri-miR-3108 and pri-miR-1251 are decreased in the various cell-types in the female SVZ compared to the male SVZ in both young and old, however (**Figure 8A and Figure 9A**). In female astrocytes, there is a decrease in pri-miR-384 expression compared to male astrocytes in the old age group (**Figure 9A**). miR-384 targets BACE1, which is involved in Alzheimer's Disease (54, 77) (**Table 1**). Since females are more prone to Alzheimer's Disease, this pri-miRNA will require investigation to determine if it contributes to the AD pathology.

Like in young data, there are few publications of target genes for these pri-miRNAs. Therefore, computational target gene prediction and their subsequent pathway enrichment was conducted using miRPath (71) (**Figure 9B and Supplementary Table 12**). Unlike in young, there was little sex-specific differences in pri-miRNAs targeting predicted target genes of pathways involved in development and differentiation. Like the young sex-specific comparisons, though, astrocytes had pri-miRNAs differentially expressed between the sexes that target genes involved in cell-cell interaction and neuroblasts had pri-miRNAs differentially expressed between the sexes that target genes involved in axon guidance (**Figure 9B and Supplementary Table 12**). Of additional intrigue, pri-miRNAs differentially expressed between the sexes in various cell-type have predicted target genes in the calcium signaling pathway, which was also came up as having differentially expressed genes between the two sexes in the old age group (**Figure 9B**). Calcium signaling thus appears to elicit further research on differences between the sexes with age in the SVZ. Together, these findings demonstrate there are few, yet important sex-specific differences in pri-miRNA expression in the aged SVZ cell-types compared to the young cell-types, which have far greater differences in pri-miRNA expression between the sexes.

Table 1: Function of Mature miRNAs Processed From pri-miRNA Transcripts

Differentially Expressed Between the Sexes in Young and Aged SVZ

miRNA	Cell types with DE of the primary transcript in Young Female SVZ compared to Young Male SVZ	Cell types with DE of the primary transcript in Old Female SVZ compared to Old Male SVZ	Cellular Processes and Gene Targets
miR-21a	↓ Expression: Neuron, Astrocyte 1, TAP, Vascular Endothelium	NA	Increases neurogenesis (45)
miR137	↑ Expression: Astrocyte 1	↓ Expression: Astrocyte 1, OPC, Mature Oligodendrocyte	Represses Msi1 targets which causes stem cell differentiation (47). Additionally has neuroprotective effects (76).
miR582	↑ Expression: Neuron, Immature Neuron, Astrocyte 1, Neuroblast, OPC, Mature Oligodendrocyte, Vascular Endothelium	NA	Represses various target genes in the apoptosis pathway(78).
miR490	↑ Expression: Neuron, TAP, Neuroblast, OPC, MFOL 1, Microglia, Pericyte, Ependymal Cell	NA	Directly targets HDAC2 (73), which is helps to regulate differentiation from a stem to a committed progenitor cell (74).
miR128-2	↑ Expression: Neuron, Immature Neuron, Astrocyte 1, Astrocyte 2, NSC, TAP, Neuroblast, OPC COP, MFOL 2, Mature Oligodendrocyte, Microglia, Pericyte, Ependymal Cell, Vascular Endothelium	NA	Directly inhibits stem cell factors such as KLF4, CSF1, NANOG, and SNAIL (79). Increases in this miRNA decreased progenitor proliferation and led to differentiation(48).

miR384	NA	↓ Expression: Neuron, Astrocyte 1, Neuroblast, COP, MFOL 2, Mature Oligodendrocyte, Pericyte	Targets BACE1, which is an important enzyme involved in development of Alzheimer's Disease (77).
miR128-1	↑ Expression: Neuron, Astrocyte 1, Astrocyte 2, NSC, TAP, Neuroblast, OPC, MFOL 2, Mature Oligodendrocyte, Microglia, Pericyte, Ependymal Cell, Vascular Endothelium	NA	Inhibits IGF-1 (72).

Table 1: Function of Mature miRNAs Processed From pri-miRNA Transcripts

Differentially Expressed Between the Sexes in Young and Aged SVZ

Column 1 indicates the mature miRNA name for primary miRNAs that are differentially expressed between cell-types in sex-specific manner in young and in aged SVZ. These miRNAs have relevant, validated target genes in literature. Column two indicates the direction of miRNA differential expression as well as which cell-types are differentially expressing these miRNAs in the young female SVZ compared to the young male SVZ. Column three indicates the direction of miRNA differential expression as well as which cell-types are differentially expressing these miRNAs in the old female SVZ compared to the old male SVZ. Column 4 describes the function or validated target genes found in literature.

Discussion

Currently, studies of sex-specific differences in the aging neurogenesis process of the SVZ are limited. Furthermore, studies of sex-specific differences in the SVZ often use methods

to create artificial phenotypes such as injection of estrogens or removal of ovaries rather than studying the baseline differences between males and females (26, 27, 80). Additionally, many of these studies focus on the effects of neurogenesis rather than studying the cell-types specific differences in gene expression between the sexes. Differences in regulatory RNAs, such as miRNAs, have had little focus in the literature in terms of sex-specific and age-associated differences in the cell-types of the SVZ. It is imperative to understand these differences in gene and miRNA expression within the young and aged SVZ cell-types since therapeutics have been proposed to use SVZ stem cells to propagate neurons to replace damaged neurons, in cases of stroke or neuronal damage, and replace oligodendrocytes in cases of demyelinating injury (81). The SVZ niche of stem cells is prime for these therapeutic opportunities since NSCs in this region are multipotent (4, 5). On the other hand, the NSCs in this region rely heavily on cell-cell signaling from neighboring cell-types, and there is a rise in dysfunction within these cell types with age (2, 3, 20, 22, 37, 39, 42, 82-87). Therefore, to make NSCs a viable therapeutic, differences in gene-expression and regulatory RNA expression, which guides gene-expression at the protein level, must be explored in both sexes and age groups, so these variables can be accounted for in using them as therapeutics in the brain. Furthermore, it is paramount to understand the basic differences in this neurogenic region between the sexes to further understand development and age-associated dysfunction in neurogenesis. In this study, we compared differences in gene-expression within the cell-types of the SVZ in both an age and sex dependent manner. This study is a first of its kind and a considerable step in uncovering sex-specific differences in age-associated gene expression guiding SVZ dysfunction, although more examination will need to be conducted **(see Future Directions and Experiments section)**.

Comparing old female cell-types in the SVZ to young female cell-types in the SVZ revealed a difference in Tsix expression with age. In the aging female cell-types, Tsix increased in expression, which is interesting since Tsix is the antagonist of Xist – the long non-coding RNA

involved in repressing the second X chromosome in females. It is vital to understand the impact of this difference in expression of Tsix since this may lead to improper dosage of genes on the X chromosome in female SVZ cell-types with age.

Differences in Wnt Signaling with Age Between the Sexes

Tcf7l2 expression was additionally decreased in every cell-type of the female SVZ with age. Tcf7l2 is activated by the Wnt/ β -catenin pathway, which helps to regulate NSC quiescence and differentiation (57, 58, 88). Ultimately, this decrease in Tcf7l2 likely results in increased commitment to glia and oligodendrocytes thus limiting neurogenesis (57, 58). Furthermore, although the male SVZ cell-types did not have a direct decrease in Tcf7l2, there was a decrease in 36 target genes of Tcf7l2 thus indicating a decrease in Tcf7l2 activation. Therefore, there may be a different program at hand controlling Wnt signaling in female and male SVZ cell-types. Perhaps increased Wnt/ Ca^{2+} is blocking activation of Tcf7l2 (89). In the male SVZ, Daam1 is decreased in expression with age. This decrease could limit signaling through non-canonical planar cell polarity Wnt signaling. Furthermore, since MAPK genes were overrepresented by differentially expressed genes with age, it will be necessary to disentangle whether this leads to increased JNK activation through the Planar Cell Polarity (Non-Canonical Wnt) pathway. This pathway controls more of the proliferation of neuroblasts and control of cerebrospinal fluid (CSF) movement, although there have been reports that this pathway can control NSC quiescence post-injury (37, 83).

pri-miRNA expression, in addition to gene expression, may also contribute to sex-specific differences in Wnt signaling. In the young SVZ, pri-miRNAs differentially expressed between the two sexes are predicted to target genes in the Wnt signaling pathway. These predictions must be validated to state the impact of miRNAs more conclusively on Wnt signaling in a sex-specific manner, however. Some of the genes that are predicted to be targeted include

the Wnt genes as well as Fzd receptors. Therefore, Wnt signaling may be differentially expressed even in the young SVZ in a sex-specific manner to regulate neurogenesis.

Differences in Calcium Signaling with Age Between the Sexes

In addition to *Tsix* and *Tcf7l2*, 87 other genes were found differentially expressed in every cell-type in the aged female SVZ compared to young. Although there were some differences among overrepresented pathways between the aged and young cell-types, there was an overall pattern showing that pathways of neurodegeneration (multiple) was among the most highly overrepresented pathway by differentially expressed genes in the aged female SVZ (**Figure 2B, Supplementary Figure S1**). Interestingly, many of the genes in the calcium signaling pathway, although the pathway itself was not enriched, appeared in several overrepresented pathways. *Calm1*, *Adcy1*, *Camk2n1*, and *Camk2a* increased in expression with age in most cell-types while *Cacna1c* and *Creb5* decreased in expression with age (**Figure 10A**). This is interesting since calcium enters through L-type voltage gated channels, such as Cav1.2 encoded by *Cacna1c*, and calcium can signal through calmodulin and *Camk2a* to phosphorylate *Creb* leading to transcription of cAMP-Response Elements (CRE) (90). Within this transcriptional dataset, we can see both increased transcription of *Camk2a* and the calcium/calmodulin dependent protein kinase II inhibitor I (*Camk2n1*). Therefore, it's unclear if *Camk2a* protein activity is increased or decreased as a result. With calmodulin (*Calm1*) and *Camk2a* increased with age yet *Creb* and *Cacna1c* levels decreased with age, so there may be less calcium actively entering cells leading to *Creb* transcription of CREs.

In addition, calcium can activate *Adcy1* leading to catalysis of ATP to cAMP. Adding another element of complexity to this model, *Adcy1* is increased in expression with age in female cell-types, and *Adcy1* functions to catalyze ATP to cAMP in the presence of calcium (91). Ultimately this leads to cAMP protein kinases (PKA) activation on *Creb* phosphorylation (91). In the brain, overexpression of ADCY1 led to increased memory and MAPK signaling (92).

MAPK signaling is essential for neurogenesis, and calcium entry from Cav1.2 and signaling through calmodulin helps to increase this MAPK signaling as well(93).

In future studies, calcium levels must be measured along with Cav1.2 levels to determine if calcium entry is impaired in the female SVZ with age. In addition, levels of Creb phosphorylation will need to be assessed and genomic wide analyses will need to be conducted to determine if genes with CREs are increased or decreased in expression in the female SVZ with age. This will ultimately determine if there is a gain in transcription of genes containing CRE elements or a loss. For example, calcium entry through Cacna1c encoded Cav1.2 is necessary for Camk2 phosphorylation of NeuroD (94, 95). Via this phosphorylation, genes regulating glial-fate determination are inhibited (95). Therefore, it will be interesting to observe whether Camk2a activity is upregulated or downregulated, ultimately. Additionally, decreased Errb4 in the female SVZ cell-types paints a picture of limited calcium entry and, likely, limited migration of neuroblasts (96, 97).

Although the genes guiding calcium signaling are similar in their differential expression, with age, between female and male cell-types of the SVZ, Cacna1c and Cacna1a are increased in expression in the male cell-types (**Figure 10B**). This indicates a likely difference in calcium stores and calcium content with age in the male SVZ cell-types. The consequences of this difference, however, must be an area of future exploration. Interestingly, estrogen, which decreases in age in the female mice, regulates the expression of CACNA1C via Creb phosphorylation (55, 63, 65). Furthermore, genes like Chrm2, which are decreased with age in the female SVZ but remain unchanged in the OF v OM comparison are regulated by estrogen as well (70).

In addition to differential expression of genes in the calcium signaling pathway with age, there are differences in pri-miRNA expression between the old female SVZ cell-types and old male SVZ cell-types that are predicted to target genes in the calcium signaling pathway. It will

be imperative to determine which pri-miRNAs are validated to target genes in this pathway, and how this effects sex-specific differences and ultimately neurogenesis with age.

Together, this data demonstrates that age-associated and sex-specific differences are at play in the calcium signaling pathway. With age, females have a decrease in estrogen circulating within the bloodstream (65). This decrease likely leads to a decrease in transcription of genes with estrogen response elements (EREs) such as *Cacna1c* (63). The ultimate result of decreased calcium entry, however, must be confirmed and characterized with functional assays within the cell-types. Perhaps, the decreased estrogen exposure in the female brain with age can explain how female and male cell-types share similar gene-expression with age.

Although most genes described changed in every single-cell type, there were genes in the calcium signaling pathway that did change depending on cell-type. Even *Cacna1c* transcription was not changed in every single SVZ cell-type in the female with age. Therefore, the interplay between cell-types with these differences in calcium content must be explored. Furthermore, pri-miRNA expression's impact on sex-specific and age-associated gene expression must be further examined to determine if there's a "one-two punch" mechanism of gene regulation, whereby some genes in a pathway are increased or decreased in expression transcriptionally while even more genes are further increased/decreased in expression by miRNA function.

In conclusion, we suggest there is limited entry of calcium based on a reduction in *Cacna1c* transcription, likely due to decreased estrogen present in the aged female blood, in the female SVZ cell-types with age. This reduction is sex-specific, and it may result increased glial production over neuroblast production from progenitors with age. Validation and mechanistic studies will be needed to determine the ultimate contribution to SVZ cell-type specific decline with age in the female and male SVZ.

Conclusions: The gene expression between the female SVZ cell-types and male SVZ cell-types becomes more similar with age. Wnt signaling and calcium signaling appear to have differences in gene expression and regulation based on differential pri-miRNA transcription of pri-miRNAs with predicted target genes within the pathways. The differences observed in calcium signaling may be the result of differences in estrogen expression. The impact of these differences between age and sex on SVZ function and age-associated dysfunction, however, are inconclusive and will be studied as a future direction.

Methods

Single-nucleus Preparation from the Male and Female SVZ

Eight 3–4-month-old male, eight 3–4-month-old female, eight 25–26-month-old male, and eight 25–26-month-old C57BL6 female mice were sacrificed in accordance with IACUC guidelines. Two SVZs constituted one sample, so this experiment had four young female, four young male, four old female, and four old male samples each. Upon dissection, we followed the same modified protocol of the 10X Single-nucleus isolation protocol (10X Genomics, CG000124, Rev A)(98) as used in chapter 2.

Single-nucleus Library Preparation and Sequencing.

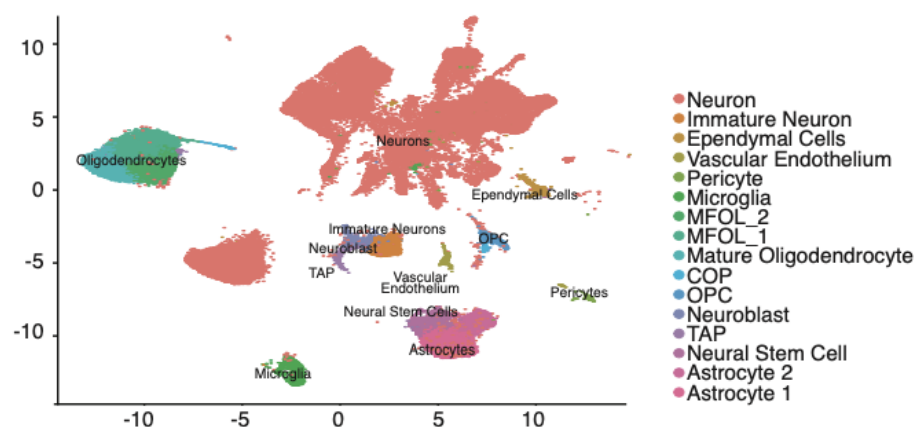
Isolated nuclei were used for library preparation using the 10X v3 3' protocol (10X Genomics, CG000183, Rev A) (99). Each library consisted of 5,000 nuclei per sample sequenced to a depth of 5,000 reads per cell (250 million reads per sample) using the HiSeq Sequencer at Genewiz.

primiReference Analysis of Young and Aged SVZ

Using primiReference, described in chapter 2, as a reference for the 10X Cell Ranger program, files were aligned and aggregated into matrices usable by Seurat (100). Using Seurat, Nuclei

were filtered, so nuclei with features between 200 and 3751.45 were subset as well as mitochondrial contamination less than 4.96%. The nuclei were clustered into 50 principal components, based on Jackstraw analysis, and reduced using UMap. The cells were labeled using two methods: the first being integration with the Allen Brain Atlas and the second being marker gene identification from published literature (101-112). Clusters identified as contamination or with few cells were removed from the analysis, and differential expression analysis was conducted on the remaining clusters between the two age and sex groups in this study using Seurat. Following differential expression analysis, a heatmap of pri-miRNAs was constructed using Morpheus (<https://software.broadinstitute.org/morpheus>) to demonstrate the significantly expressed ($p < .05$, non-parametric Wilcoxon rank sum test) pri-miRNAs within each cell type with age or sex-specific differences. These primary microRNAs were input into miRPath using DIANATOOLS(71) in the same manner described in chapter 2. For differentially expressed genes between age and sex, ClusterProfiler was used to conduct overrepresentation analysis, visualize concept gene networks, and visualize impact on pathways via the pathview package (53, 113). Furthermore, Qiagen Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>) was used to compare the number of genes that were similar or unique in comparisons using the compare function (56). Ggplot2 was used to visualize the overrepresentation analysis (114). Biorender.com was used to create schematics to represent the calcium signaling pathway activating CREB.

1A



1B

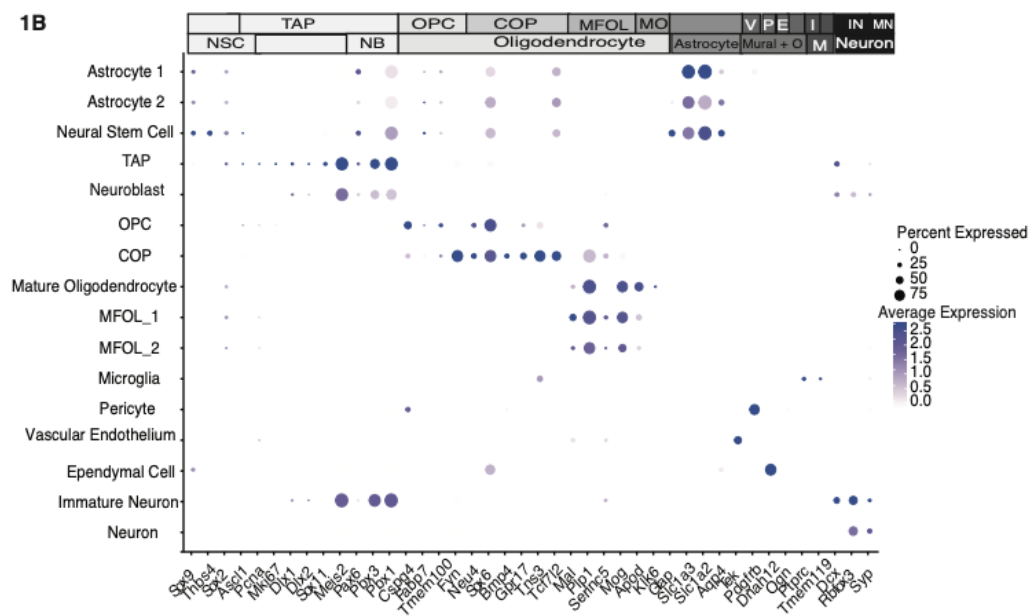


Figure 1: Cellular Content of the Aging SVZ in Males and Females: **Figure 1A:** The UMap of young and aged male and female SVZ is shown. There are 14 unique cell-types with additional astrocyte and myelin forming oligodendrocyte (MFOL) clusters. **Figure 1B:** The cell-type specific markers are shown along with gene-expression of those markers per cell-type. NSC stands for Neural Stem Cell, TAP for transiently amplifying progenitor, NB for neuroblast, OPC for oligodendrocyte progenitor cell, COP for committed oligodendrocyte progenitor cell, MFOL for myelin forming oligodendrocyte, MO for mature oligodendrocyte, V for vascular endothelium, P for pericyte, E for ependymal cell, I for immune cell, M for microglia, IN for immature neuron, and N for neuron. The shade of blue indicates increased expression while the size of the dot indicates the number of cells expressing this gene.

2A

Age Associated Changes in Gene Expression in the Female SVZ

	Genes Increased in Expression OF v YF	Genes Decreased in Expression OF v YF
Astrocyte 1	209	132
Astrocyte 2	232	150
NSC	216	150
TAP	227	171
Neuroblast	200	184
OPC	196	136
COP	206	165
MFOL 1	223	171
MFOL 2	217	162
Mature Oligodendrocyte	187	153
Microglia	204	118
Pericyte	213	147
Ependymal Cell	209	164
Vascular Endothelium	208	137

2B: Overrepresented Pathways of Differentially Expressed Genes in the NSCs and Progenitors of the Female SVZ

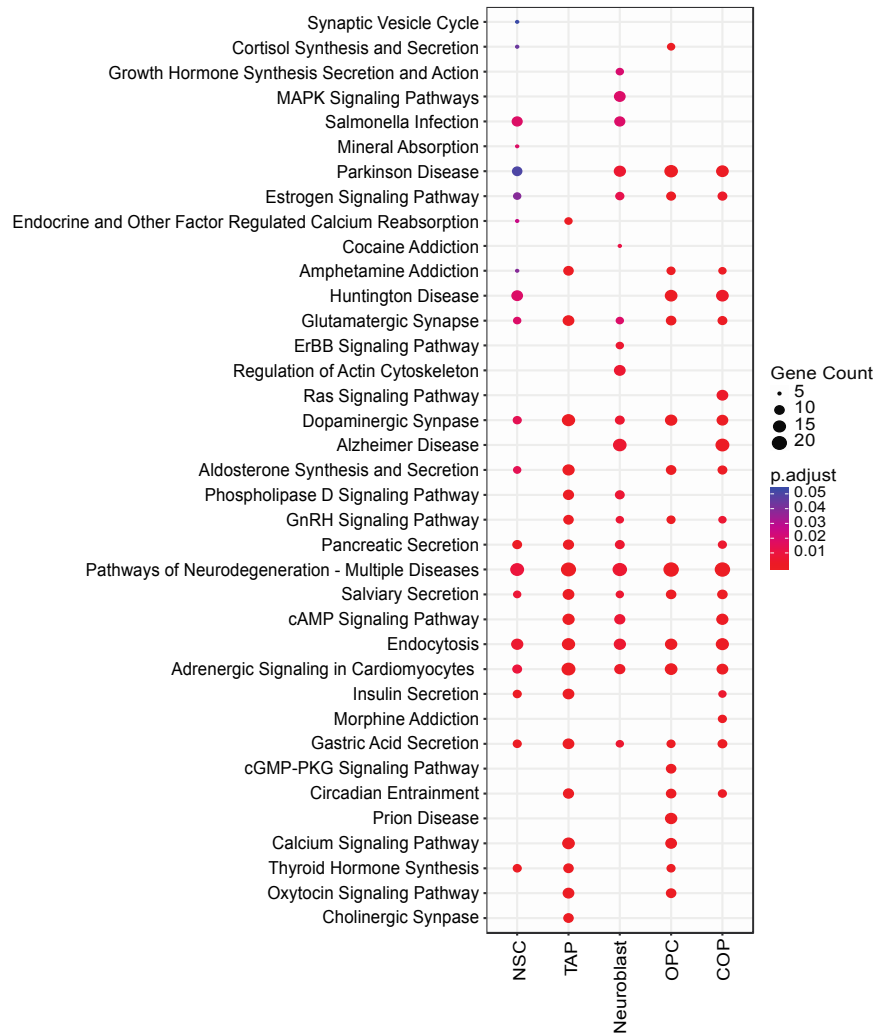


Figure 2: Age-Associated Changes in Gene Expression within the Female SVZ. Figure 2A

The number of genes increased or decreased in old female (OF) compared to young female (YF) per cell-type is shown. **Figure 2B:** A plot depicting pathways, on the Y axis, in which differentially expressed genes, with age, are overrepresented between old female and young female NSCs and their progenitors is shown. On the X axis, the cell-type is indicated. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The top twenty significant genes per pathway are represented.

3A

Age Associated Changes in Gene Expression in the Male SVZ

	Genes Increased in Expression OM v YM	Genes Decreased in Expression OM v YM
Astrocyte 1	239	314
Astrocyte 2	227	322
NSC	258	337
TAP	271	361
Neuroblast	264	328
OPC	285	380
COP	211	282
MFOL 1	225	306
MFOL 2	244	331
Mature Oligodendrocyte	235	297
Microglia	222	295
Pericyte	262	362
Ependymal Cell	238	292
Vascular Endothelium	276	343

3B: Overrepresented Pathways of Differentially Expressed Genes in the NSCs and Progenitors of the Male SVZ

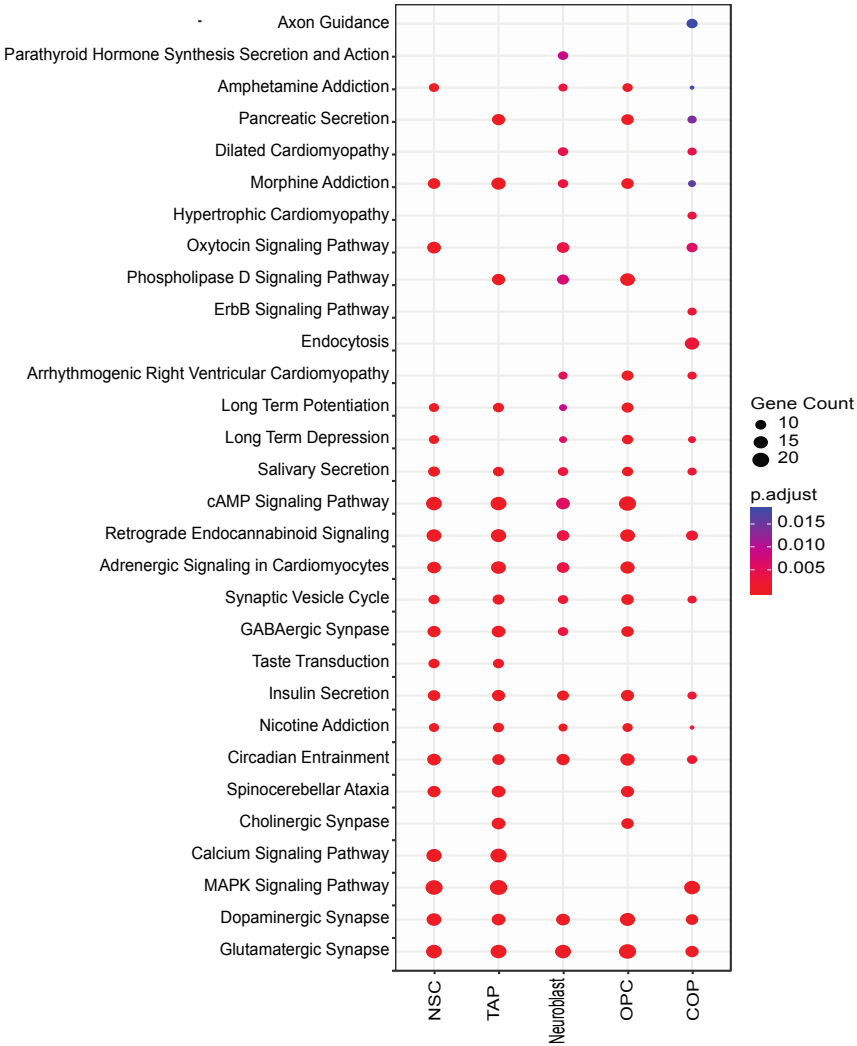
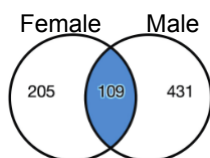


Figure 3: Age-Associated Changes in Gene Expression within the Male SVZ. Figure 3A

The number of genes increased or decreased in old male (OM) compared to young male (YM) per cell-type is shown. **Figure 3B:** A plot depicting pathways, on the Y axis, in which differentially expressed genes, with age, are overrepresented between old male and young male NSCs and their progenitors is shown. On the X axis, the cell-type is indicated. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The top twenty significant genes per pathway are represented.

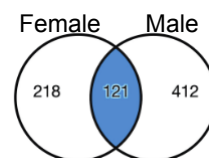
4A
Differential Expression of Genes
with Age in NSCs



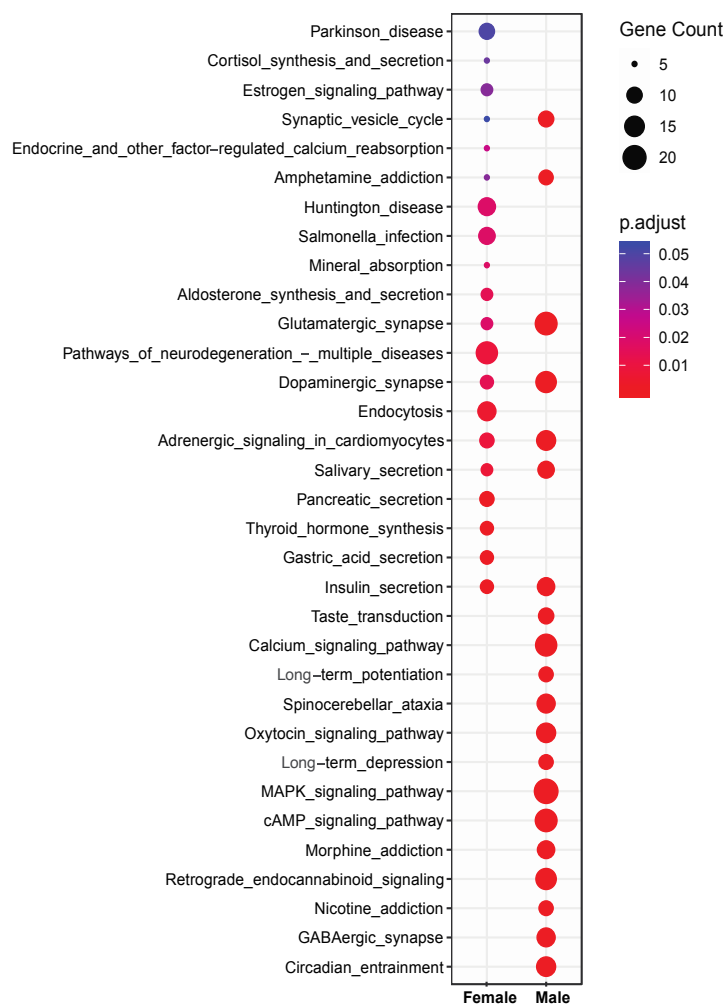
4B
Differential Expression of Genes
with Age in TAPs



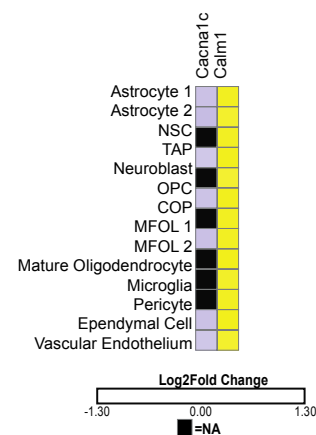
4C
Differential Expression of Genes
with Age in Neuroblasts



4D Overrepresentation Analysis of Differentially Expressed Genes, with Age, in NSCs



4E Differential Expression of Cacna1c and Calm1 with Age in the Female SVZ



4F Differential Expression of Cacna1c and Calm1 with Age in the Male SVZ

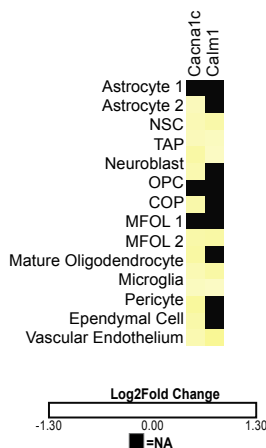


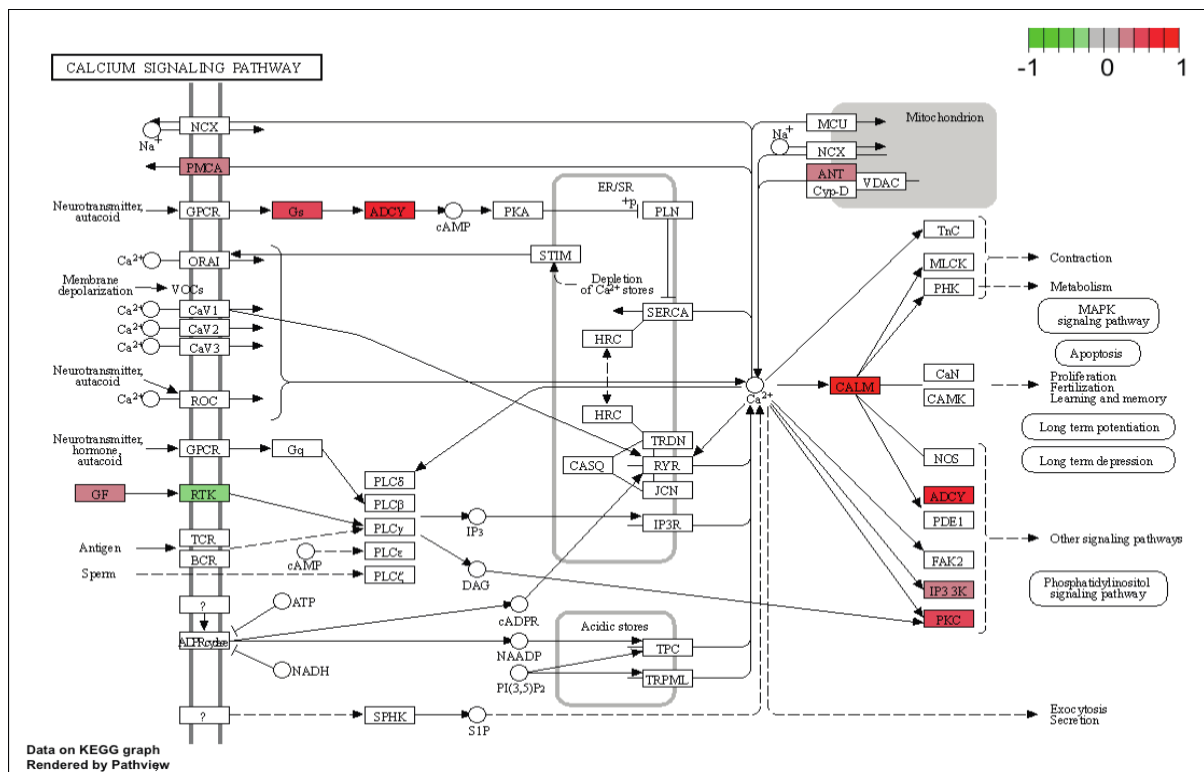
Figure 4: Sex-Specific Differences in Age Associated Gene Expression in the Female and

Male SVZ. Figures 4A-4C: Venn diagrams created with Qiagen's IPA show the number of genes differentially expressed with age in female only, male only, and overlapping age-associated changes in gene expression between the two sexes for NSCs, TAPs, and

Neuroblasts. **Figure 4D:** A plot depicting pathways, on the Y axis, in which differentially expressed genes, with age, are overrepresented in female NSCs and male NSCs with age. On the X axis, the sex in which the comparison between ages took place is shown. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The pathways represented are the top twenty most significant pathways differentially expressed with age in both the female and male NSC groups.

Figure 4E: The log₂ fold change of *Cacna1c* and *Calm1* are depicted per cell type with age in the female SVZ. Yellow indicates an increase with expression in age while blue indicates a decrease in expression with age. These two genes were selected based on their appearance in many different KEGG pathways and differential expression in most cell-types. **Figure 4F:** The log₂ fold change of *Cacna1c* and *Calm1* are depicted per cell type with age in the male SVZ. Yellow indicates an increase with expression in age while blue indicates a decrease in expression with age. These two genes were selected based on their appearance in many different KEGG pathways and differential expression in most cell-types.

5A: Differential Expression of Genes in Calcium Signaling in Female NSCs with Age



5B: Differential Expression of Genes in Calcium Signaling in Male NSCs with Age

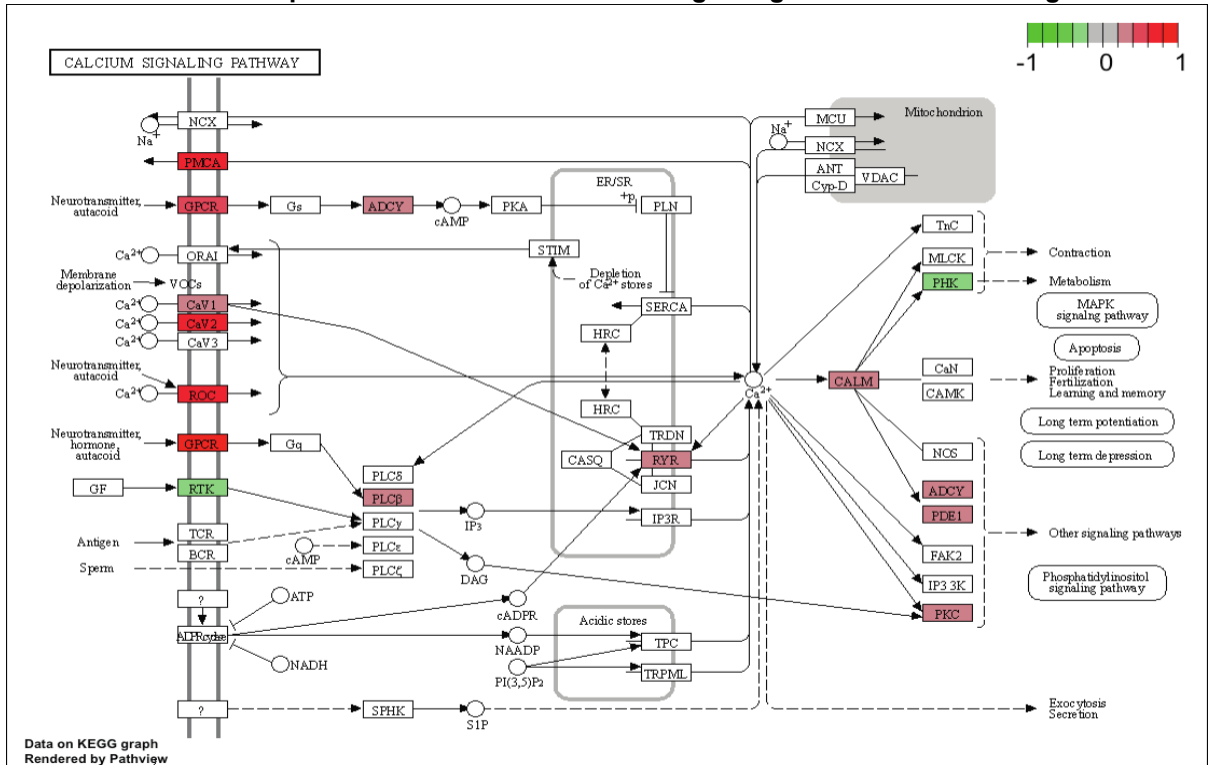


Figure 5: Differences in Calcium Signaling with Age in Female and Male NSCs. Figure

5A: The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data from the female NSCs with age. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview. GF (VEGF growth factor), PMCA (Atp2b1), Gs (Gnas), ADCY (Adcy1), ANT (Slc25a4), and PKC (Prkcb) increase with age, while RTK (ErbB4) decreases with age in female NSCs. **5B:** The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data from the male NSCs with age. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview. Cav1 and Cav2 (Cacna1c and Cacna1a, respectively), GPCR (Chrm3), PMCA (Atp2b1), ROC (Grin1), ADCY (Adcy1), PLCB (Plcb1), CALM (Calm1), Ryr (Ryr2), PDE (Pde1a), and PKC (Prkcb) increase with age, while RTK (Fgfr2) and PHK (Phkg1) decrease with age in male NSCs.

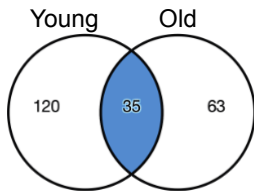
6A

Sex-Specific Differential Gene Expression in the Young and Old SVZ

	Genes Only Differentially Expressed in Young Female Compared to Young Male	Genes DE in Both	Genes Only Differentially Expressed in Old Female Compared to Old Male
Astrocyte 1	146	32	32
Astrocyte 2	117	37	32
NSC	120	35	63
TAP	145	39	50
Neuroblast	161	18	38
OPC	166	41	49
COP	119	37	48
MFOL 1	102	18	46
MFOL 2	141	33	47
Mature Oligodendrocyte	109	23	56
Microglia	173	21	23
Pericyte	159	43	53
Ependymal Cell	141	29	48
Vascular Endothelium	140	25	33

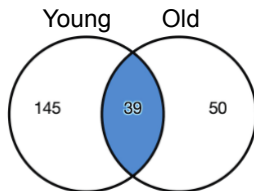
6B

Sex-Specific Differential Expression in NSCs



6C

Sex-Specific Differential Expression in TAPs



6D

Sex-Specific Differential Expression in Neuroblasts



6E Overrepresentation Analysis of Differentially Expressed Genes, by Sex, in TAPs

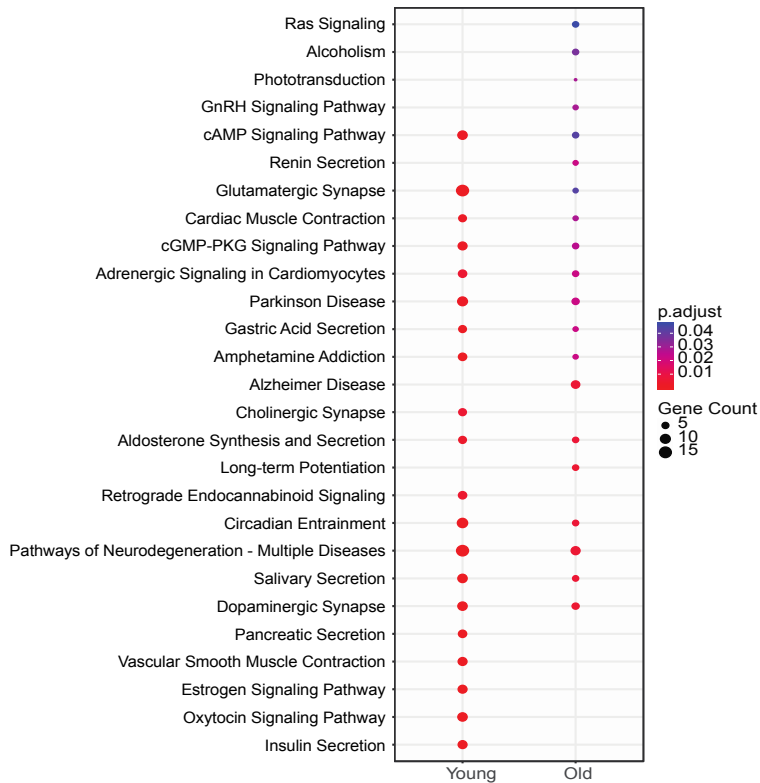
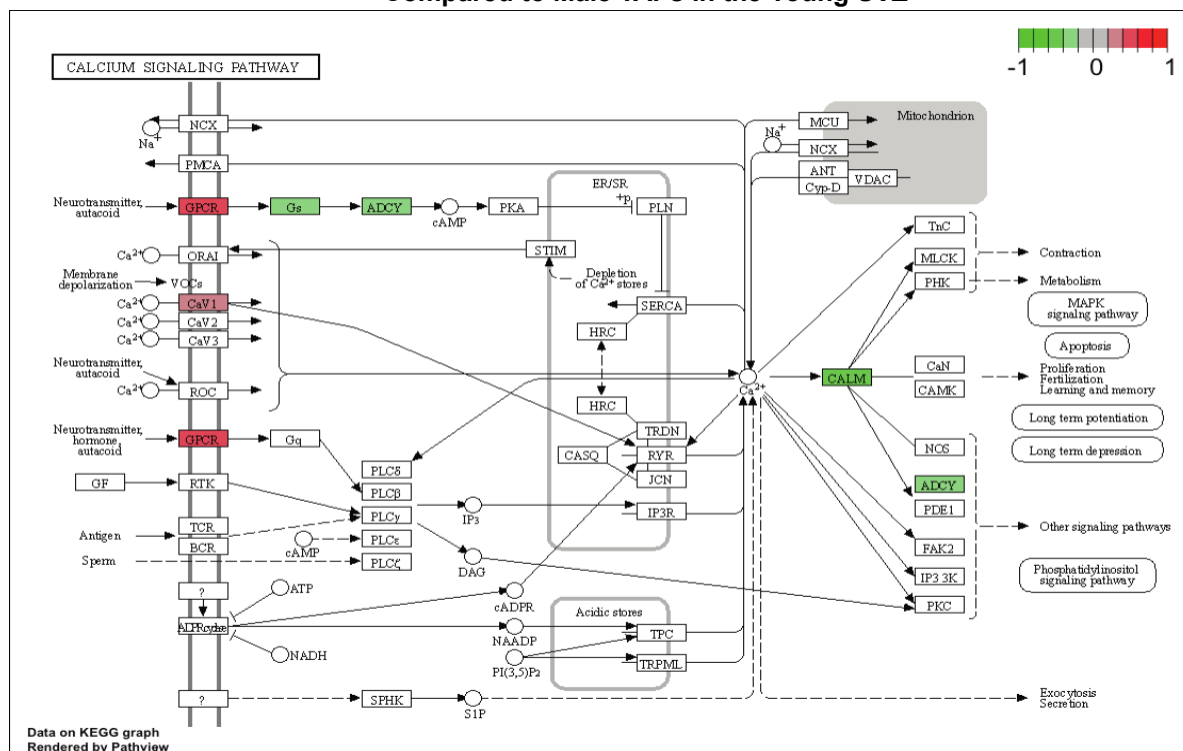


Figure 6: Sex-Specific Differences in Gene Expression in Young and Aged SVZ Cell-

Types. Figure 6A: Genes were compared between young female and young male (YF v YM) and old female and old male (OF v OM). These differences were classified as unique to the young sex-specific comparison on the left most column, shared between both old and young sex-specific comparisons in the middle column, and unique to the old sex-specific comparison in the right column. **Figure 6B-6D:** Venn diagrams of gene expression differences between young and old sex-specific comparisons in NSCs, TAPs, and neuroblasts highlight the lack of shared differential gene expression between young and aged comparisons. **Figure 6E:** A plot depicting pathways, on the Y axis, in which differentially expressed genes, between female and male, are overrepresented in TAPs from young animals and TAPs from aged animals. On the X axis, the age of the sex-specific comparison is shown. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The pathways represented are the top twenty most significant pathways differentially expressed between female and male TAPs. TAPs were selected to be used as a representative cell-type since they were the only cell-type with twenty significantly overrepresented pathways in the aged comparison.

7A: Differential Expression of Genes in Calcium Signaling in Female TAPs Compared to Male TAPs in the Young SVZ



7B: Differential Expression of Genes in Calcium Signaling in Female TAPs Compared to Male TAPs in the Old SVZ

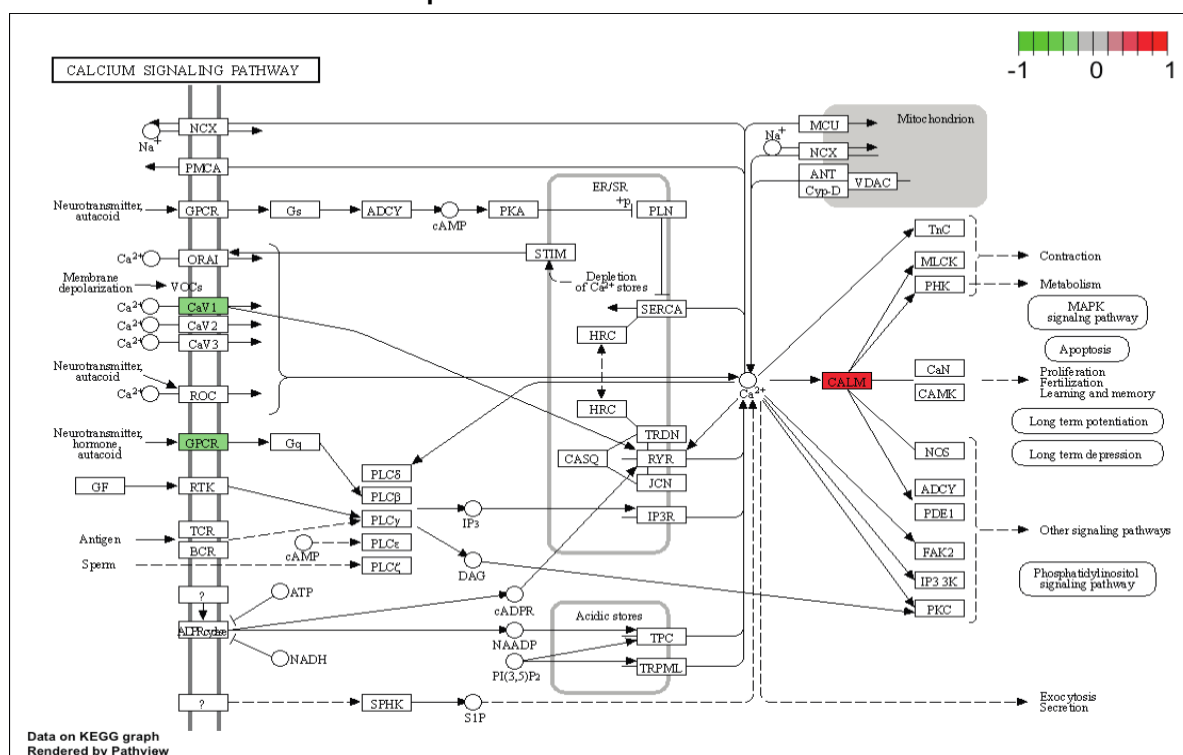
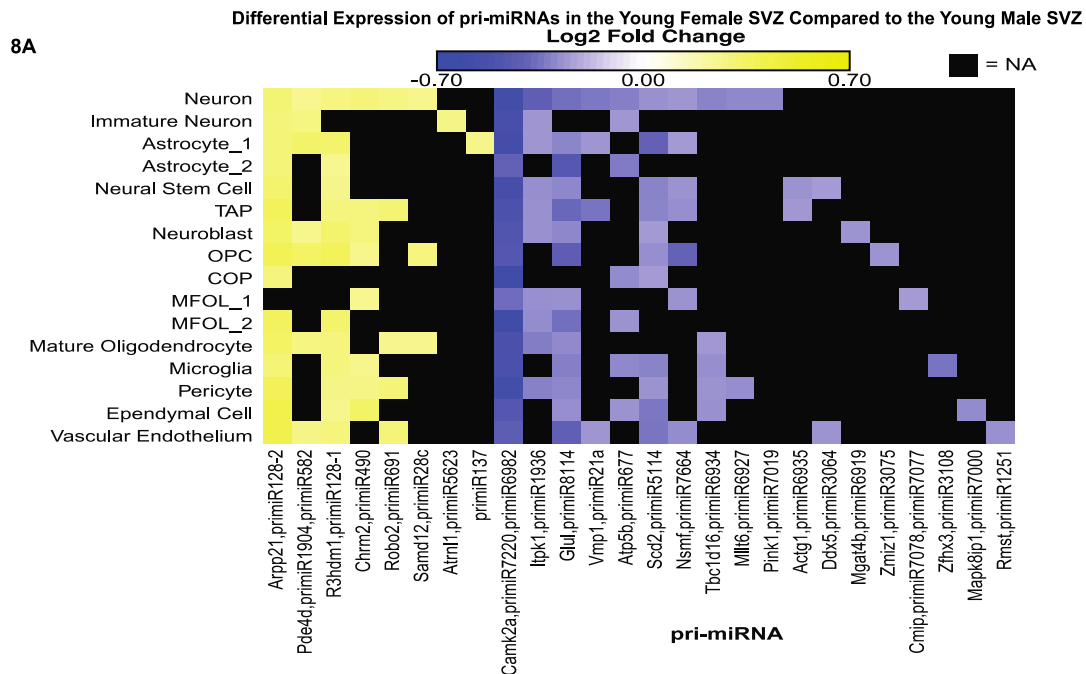


Figure 7: Sex-Specific Differences in Calcium Signaling in Young and Aged TAPs. 7A:

The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data between young female and young male TAPs. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview. CaV1 (Cacna1c) and GPCR (Chrm2 and Chrm3) are increased in expression in the young female TAPs while Gs (Gnas), ADCY (Adcy1), and CALM (Calm1) are decreased in expression in the female TAPs compared to males. **7B:** The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data between young female and young male TAPs. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview. CALM (Calm1) is more highly expressed in old female TAPs than male TAPs while CaV1 (Cacna1c) and GPCR (Grm1) have lower expression in old female TAPs compared to male TAPs.



8B

Pathways of Predicted Target Genes of pri-miRNAs With Differential Expression in the Young Female SVZ Compared to the Young Male SVZ

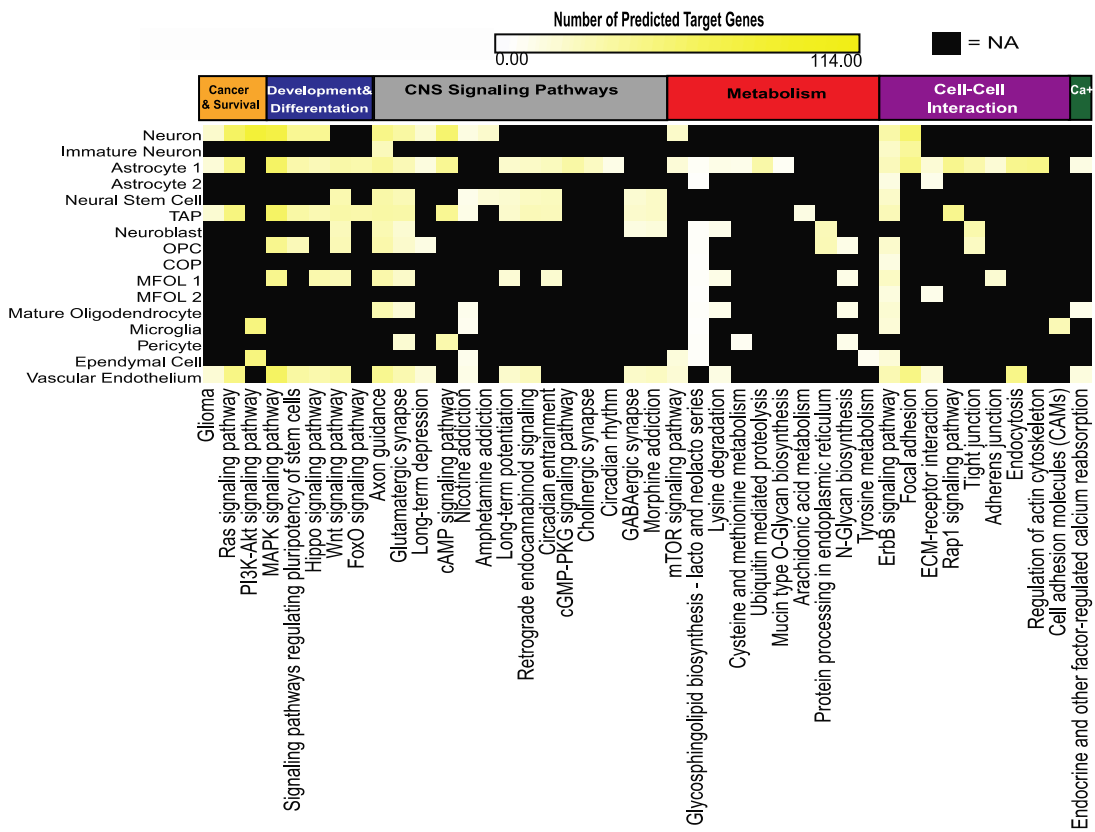
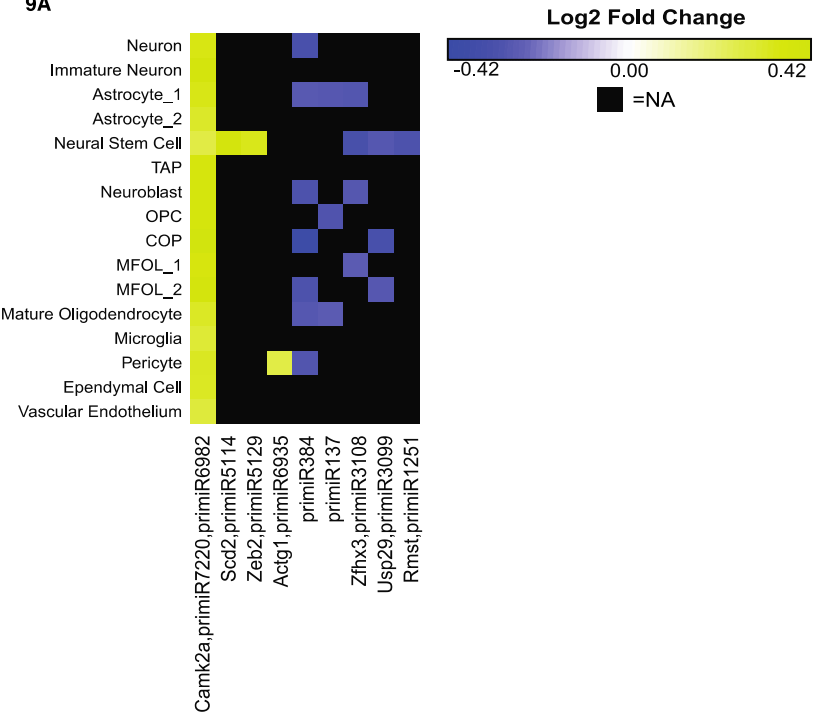


Figure 8: Sex-Specific Differential Expression of pri-miRNAs and the Pathways of Their Target Genes in the Young SVZ: **Figure 4A:** primary miRNAs with significant differences in expression between young female and young male SVZ ($p < .05$), per cell-type are shown by heatmap. Those increased in expression in the female SVZ are shown in yellow, with color depicted by the log fold-change while primary miRNAs decreased in expression in the female SVZ compared to the male are shown in blue. Black cells represent cell-types that have no change in expression with age of a particular primary miRNA that other cell-types are shown to have changing significantly. **Figure 4B:** The pathways of predicted target genes of differentially expressed pri-miRNAs, per cell-type, are depicted by the number of genes predicted to be targeted per pathway. The KEGG pathways depicted in this heatmap belong to the general categories of cancer and survival, development and differentiation, CNS signaling pathways, cell-cell interaction, and calcium signaling. All other pathways can be found in Supplementary Table 10. Yellow is used to depict the number of predicted target genes enriched per KEGG pathway of primary miRNAs changing with age

9A Differential Expression of pri-miRNAs in the Old Female SVZ Compared to the Old Male SVZ



9B Predicted Target Genes of pri-miRNAs with Differential Expression in the Old Female SVZ Compared to the Old Male SVZ

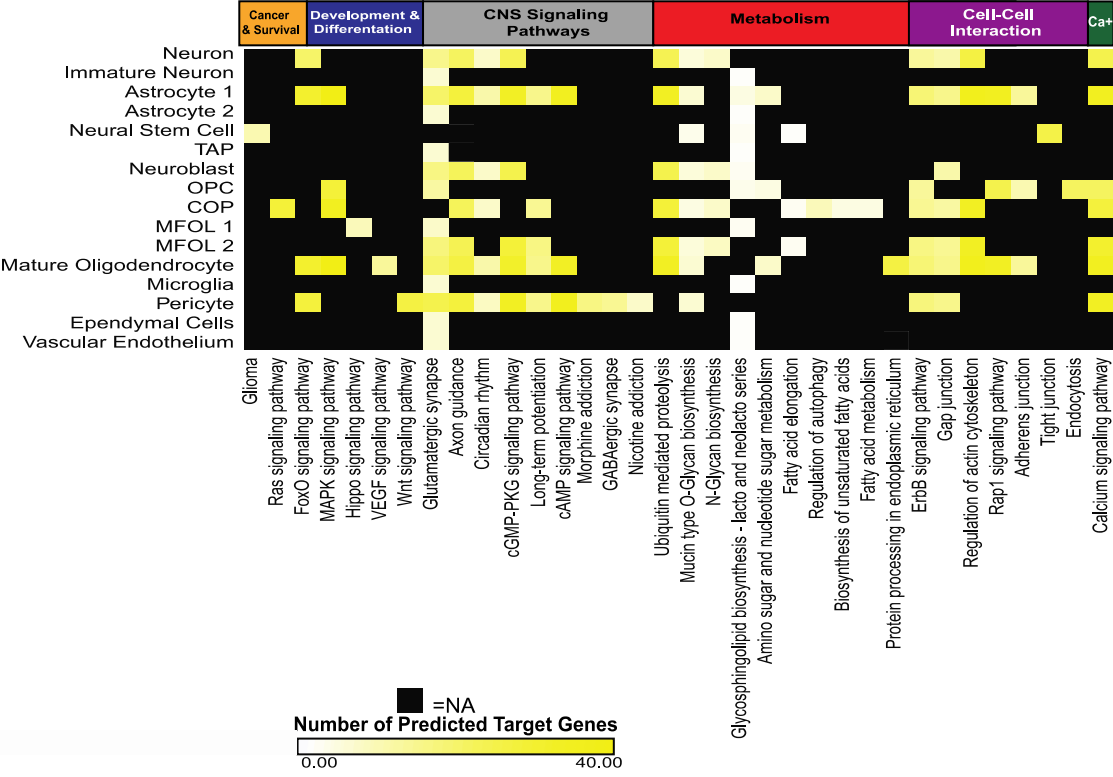
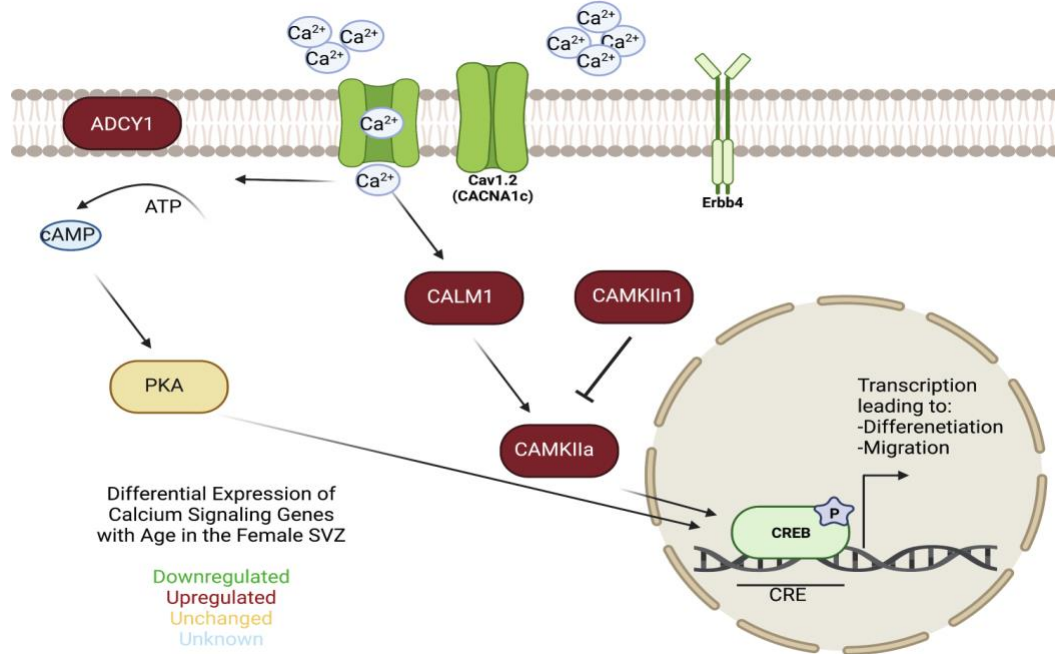


Figure 9: Sex-Specific Differential Expression of pri-miRNAs and the Pathways of Their Target Genes in the Aged SVZ: **Figure 4A:** primary miRNAs with significant differences in expression between old female and old male SVZ ($p < .05$), per cell-type are shown by heatmap. Those increased in expression in the female SVZ are shown in yellow, with color depicted by the log fold-change while primary miRNAs decreased in expression in the female SVZ compared to the male are shown in blue. Black cells represent cell-types that have no change in expression with age of a particular primary miRNA that other cell-types are shown to have changing significantly. **Figure 4B:** The pathways of predicted target genes of differentially expressed pri-miRNAs, per cell-type, are depicted by the number of genes predicted to be targeted per pathway. The KEGG pathways depicted in this heatmap belong to the general categories of cancer and survival, development and differentiation, CNS signaling pathways, cell-cell interaction, and calcium signaling. All other pathways can be found in Supplementary Table 11. Yellow is used to depict the number of predicted target genes enriched per KEGG pathway of primary miRNAs changing with age

10A: Differential Expression of Calcium Signaling Genes with Age in the Female SVZ



10B: Differential Expression of Calcium Signaling Genes with Age in the Male SVZ

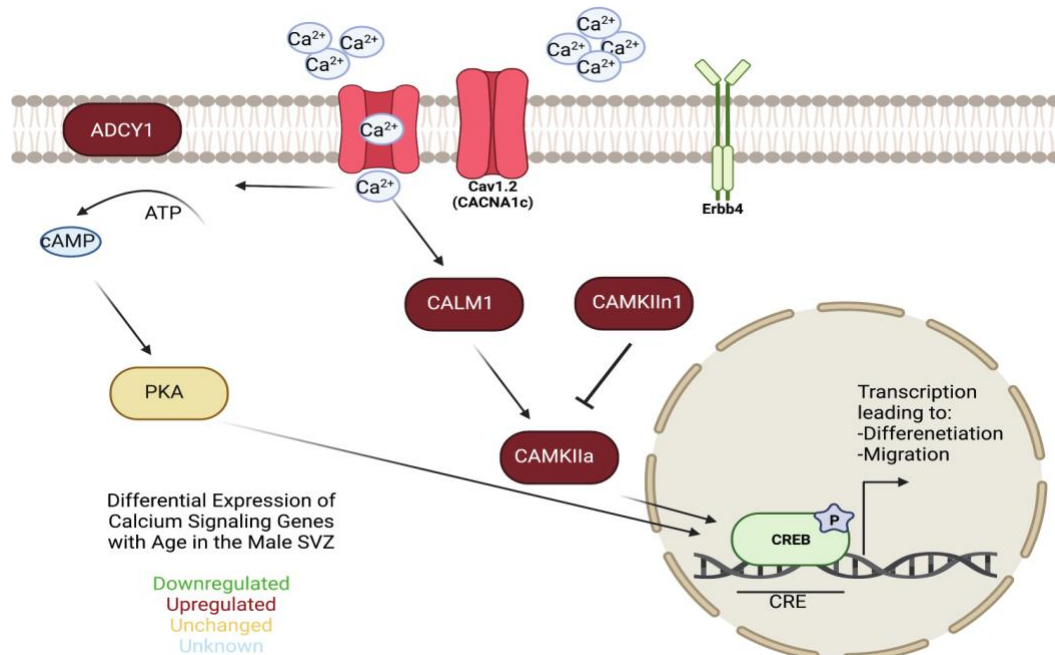


Figure 10: Models of Age and Sex Specific Differences in the Calcium Signaling Pathway

in the SVZ. Figure 10A: A schematic showing the differences in gene expression of calcium signaling/CREB transcription factor binding is shown for the aged female SVZ cell-types compared to the young. Red indicates increased expression with age, green indicates decreased expression with age, yellow indicates no change, and blue indicates unknown change with age. Cacna1c and Erbb4 import calcium while CamkIIa can phosphorylation CREB via Calm1 activation. Additionally, Adcy1 can convert calcium to cAMP and signal through PKA for Creb phosphorylation. Creb phosphorylation results in transcription of genes regulating differentiation and migration of cells in the SVZ. **Figure 10B:** A schematic showing the differences in gene expression of calcium signaling/CREB transcription factor binding is shown for the aged male SVZ cell-types compared to the young. Red indicates increased expression with age, green indicates decreased expression with age, yellow indicates no change, and blue indicates unknown change with age.

Figure S1: Overrepresented Pathways of Differentially Expressed Genes in the Cell-Types of the Female SVZ

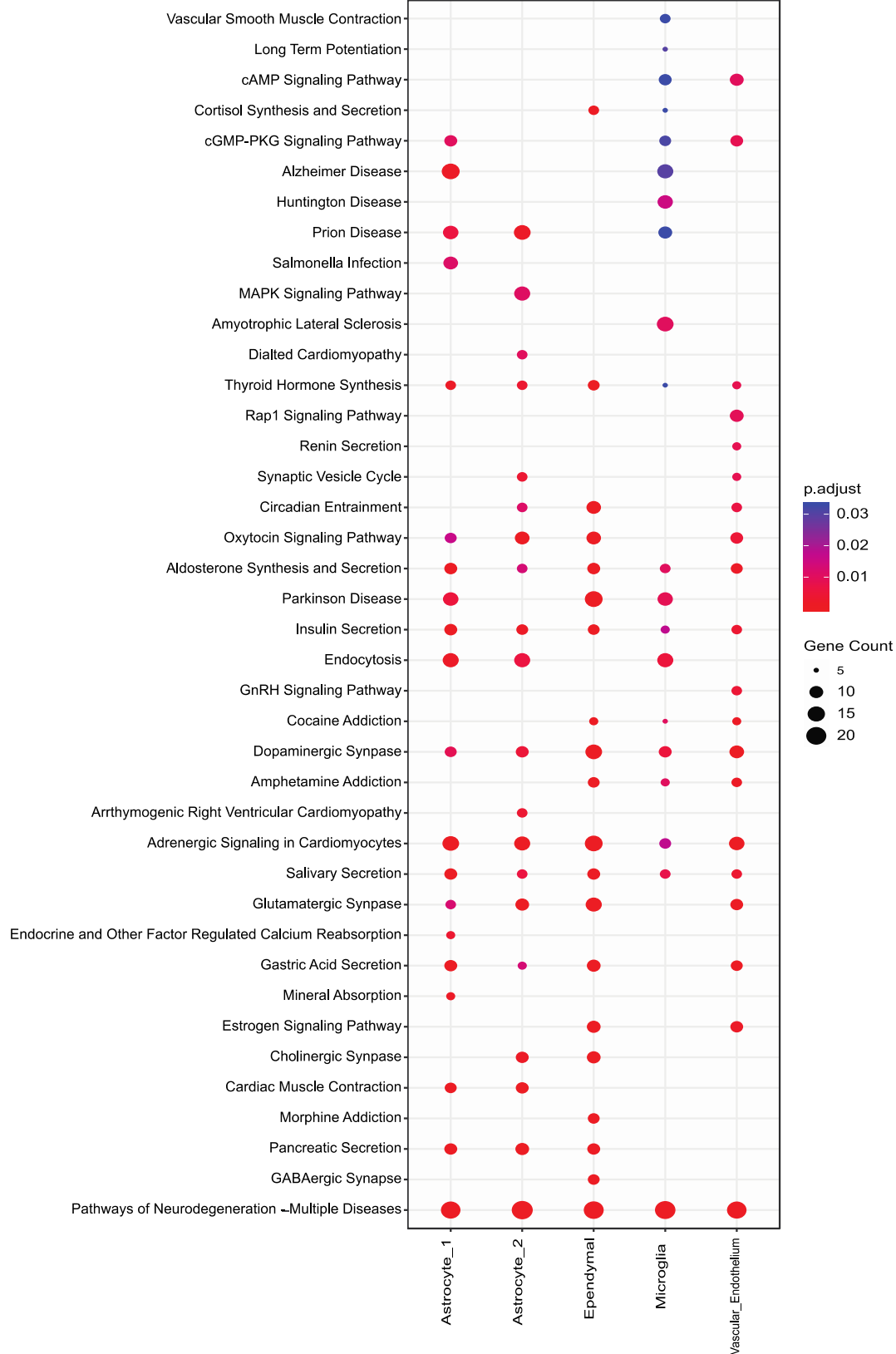


Figure S1: Overrepresented Pathways of Differentially Expressed Genes in the Cell Types of the Female SVZ. A plot depicting pathways, on the Y axis, in which differentially expressed genes, with age, are overrepresented between old female and young female astrocytes, ependymal cells, microglia, and vascular endothelium are shown. On the X axis, the cell-type is specified. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The top twenty significant genes per pathway are represented.

Figure S2: Overrepresented Pathways of Differentially Expressed Genes in the Cell-Types of the Male SVZ

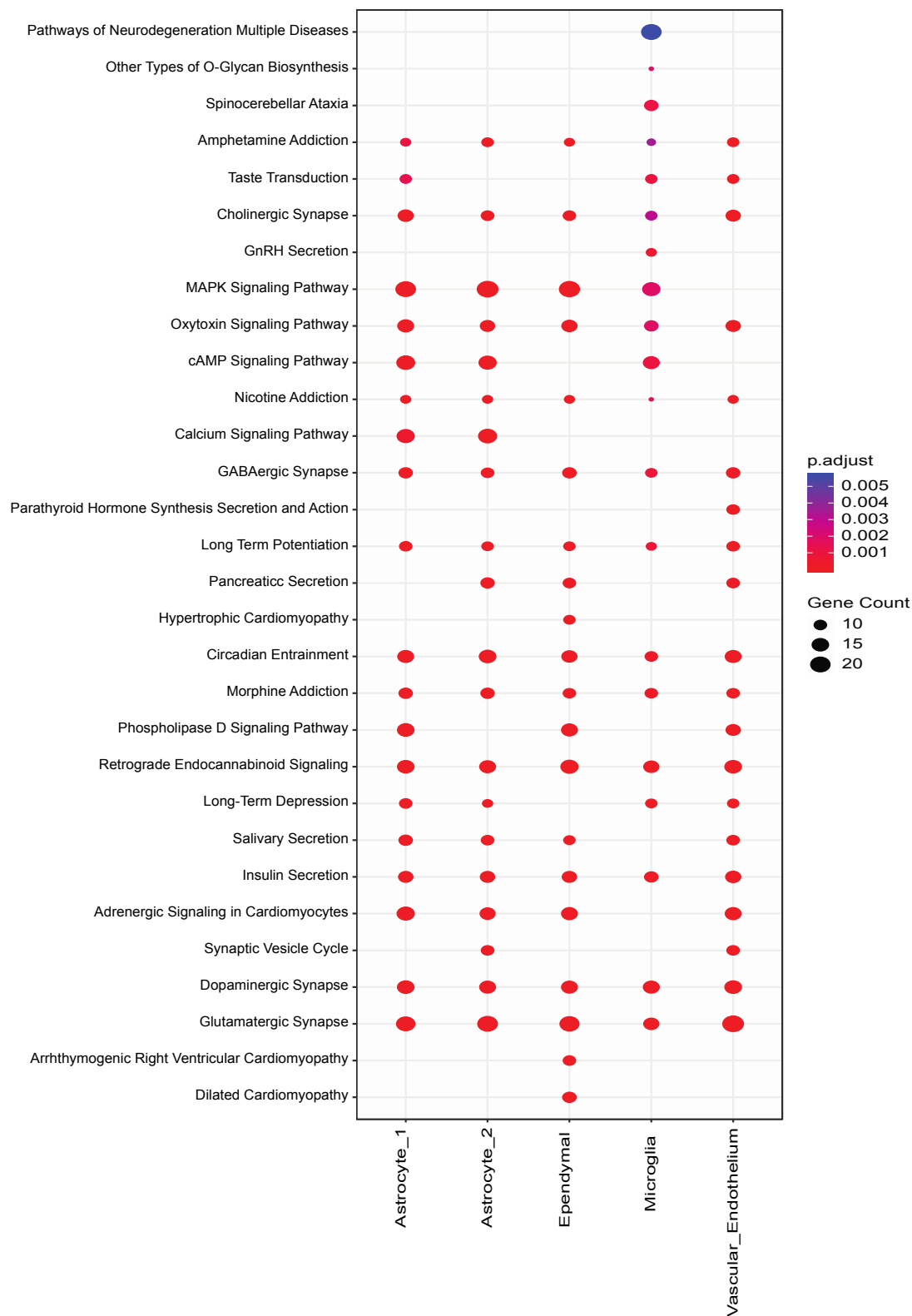
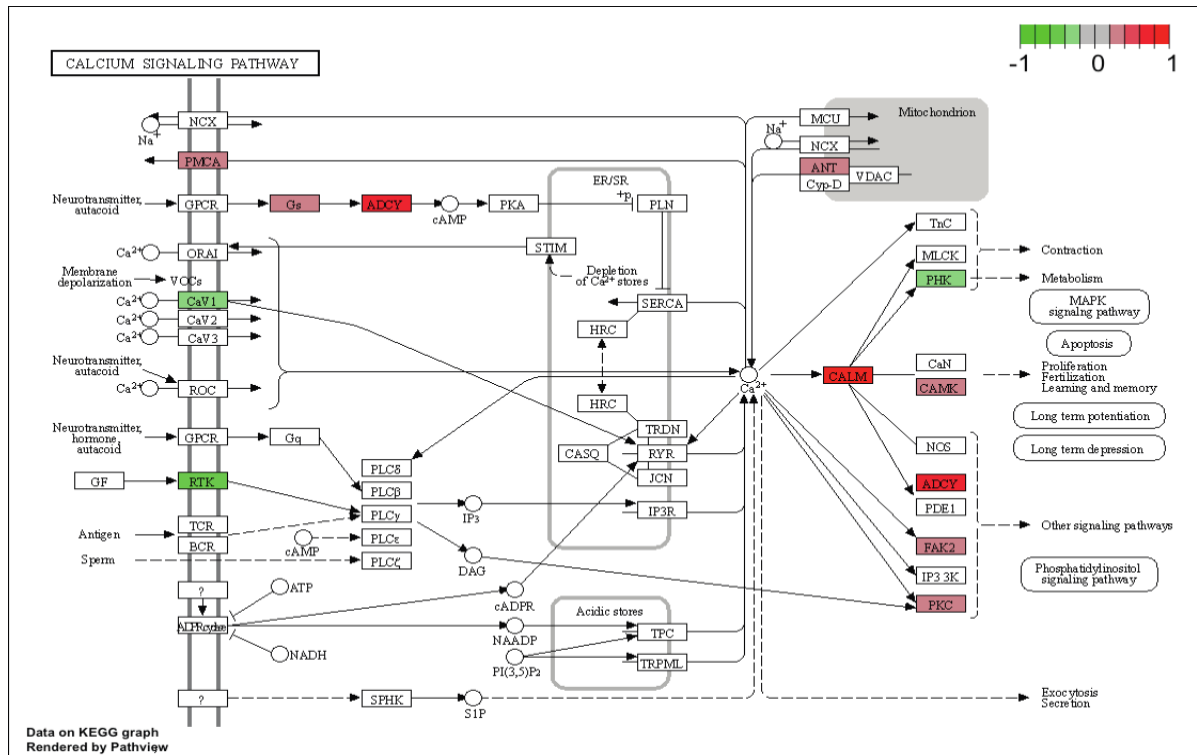


Figure S2: Overrepresented Pathways of Differentially Expressed Genes in the Cell Types of the Male SVZ. A plot depicting pathways, on the Y axis, in which differentially expressed genes, with age, are overrepresented between old male and young male astrocytes, ependymal cells, microglia, and vascular endothelium are shown. On the X axis, the cell-type is specified. The size of the dot indicates the number of differentially expressed genes found in the pathway while the color of the dot indicates the adjusted p-value. Red dots indicate a lower p-value than dots shown in shades of blue, however all are significant. The top twenty significant genes per pathway are represented.

S3A: Differential Expression of Genes in Calcium Signaling in Female TAPs with Age



S3B: Differential Expression of Genes in Calcium Signaling in Male TAPs with Age

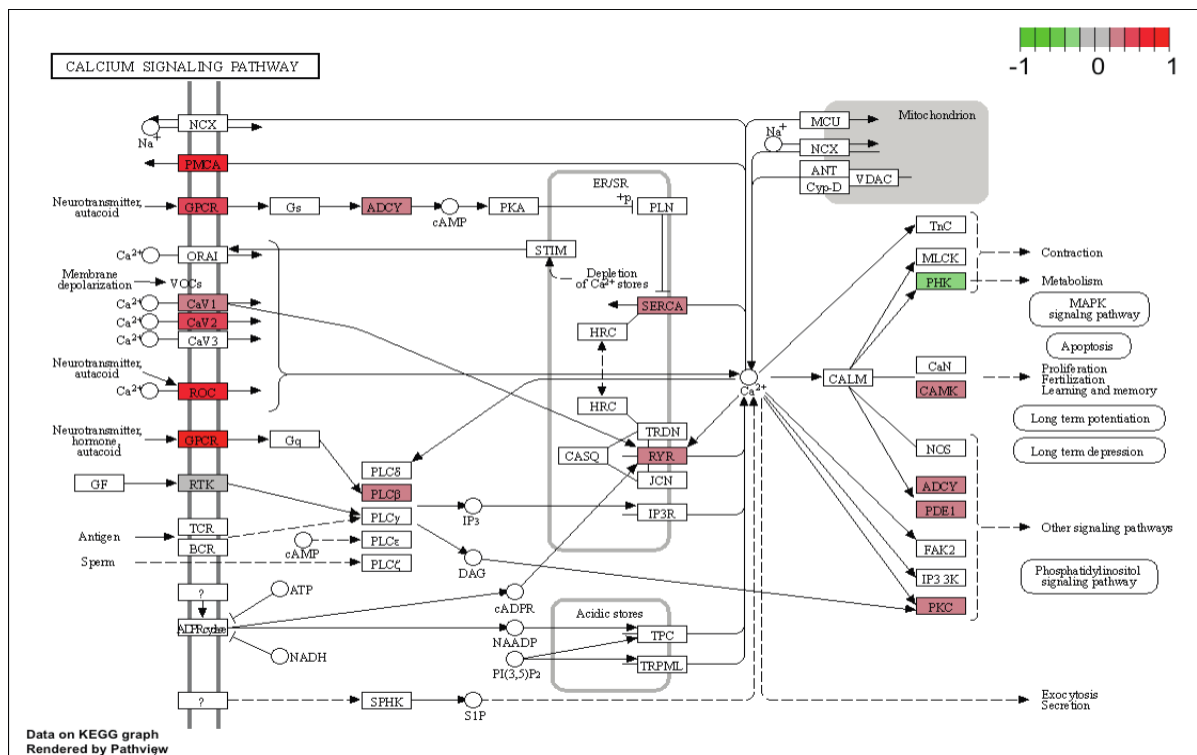


Figure S3: Differentially Expressed Genes in the Calcium Signaling Pathway of TAPs with

Age. Figure S3A: The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data from the female TAPs with age. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview.

PMCA (Atp2b1), Gs (Gnas), ADCY (Adcy1), ANT (Slc25a4), CALM (Calm1), FAK2(Ptk2b) and PKC (Prkcb), and CAMK (Camk2a) increase with age, while CaV1 (Cacna1c), RTK (ErbB4), and (Phkg1) decrease with age in female NSCs.

5B: The KEGG Pathway for Calcium Signaling (mmu04020) is overlaid with differential expression data from the male NSCs with age. Red color indicates increased expression of the gene with age while green indicates decreased expression with age. This figure was made using Pathview. Cav1 and Cav2 (Cacna1c and Cacna1a, respectively), GPCR (Chrm3), PMCA (Atp2b1), ROC (Grin1), ADCY (Adcy1), PLCB (Plcb1), Ryr (Ryr2), SERCA (Atp2a2), PDE (Pde1a), CAMK (Camk2a), and PKC (Prkcb) increase with age while PHK (Phkg1) decreases with age in male NSCs.

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Chapter 4:
Discussion

pri-miRNA Expression Can Be Elucidated from Single-Nucleus Sequencing Datasets

Single-nucleus sequencing technology allows for the elucidation of gene expression, on a single-cell level, for tissue-types for which single-cell sequencing is more difficult. Tissues such as muscle and organs such as the brain face difficulties in single-cell sequencing library preparation due to their long, fibrous cell-types, which may become sheared within the fluidics device used to conduct the preparation from time to time. Therefore, single-nucleus technology allows for elucidation of transcripts within the nuclei of these cell-types therefore bypassing the possibility of disrupting the fluidics device used to conduct the preparation. Single-nucleus sequencing technology, however, must account for lower reads, lack of pre-mRNA processing, and higher turnover compared to single-cell sequencing (1-4). On the other hand, additional studies have shown that single-cell and single-nucleus studies do have an overall agreement in expression(3, 4).

For both technologies, there are some common shortcomings. One pitfall of both techniques is the that the disaggregation or dissociation of tissue may change nascent gene expression during nuclei and cell isolation. Conversely, the agreement between single-nucleus and single-cell data, which uses different isolation techniques and times, demonstrates this may be a minor pitfall (3, 4). Future studies will be needed to address if spatial single-cell sequencing, a new technique which does not call for disaggregation of tissue, reflects single-cell and single-nucleus sequencing data as well.

A shortcoming of both technologies, however, is the library preparation requiring size-selection of transcripts used for sequencing. By limiting transcripts under 150 base-pairs, both single-cell and single-nucleus technologies limit the ability to characterize and understand small regulatory RNA gene expression on a cell-type specific basis. Ignoring small regulatory RNA transcripts, such as miRNAs, an entire area of research and understanding of gene regulation and expression is ignored.

To this end, we developed primiReference to profile pri-miRNA expression within single-nucleus datasets. Since pre-miRNA is only ~70 nucleotides in length while mature miRNA is only 22-24 nucleotides in length and only found in the cytoplasm, profiling pri-miRNAs, which are several kilobases long, allows for a first step in determining small RNA expression in a cell-type specific manner (5, 6). Through development of this reference, we were able to determine, in a cell-type specific manner, which pri-miRNAs were changing in expression – with age – in the male SVZ.

Through use of primiReference, differential expression of pri-miRNAs which effect development and differentiation, among other pathways, were characterized on a cell-type specific basis in the aged SVZ compared to young. Although discussed in further detail in the section below, the profiling of these pri-miRNAs elucidated another level of gene expression control that may contribute to age-associated SVZ dysfunction and provides targets for future therapeutics. This is just one, powerful application of this reference. Using a publicly available single-nucleus sequencing dataset of 5XFAD cortex compared to wild-type age-matched cortex, the differential expression of pri-miRNAs per cell-type in an Alzheimer's Disease mouse model were characterized (7). The mature forms of pri-miR-219a-2 and pri-miR-124-1 are known to decrease in Alzheimer's Disease. Using primiReference, the overall specific cell-types and specific subtypes of neurons having decreased expression of either or both of these pri-miRNAs were elucidated. Since miR-219a-2 is implicated in successful oligodendrogenesis, myelination, and remyelination, and deficits in miR-219a-2 expression are associated in demyelination and reduced oligodendrogenesis, this serves as an important therapeutic target (8-15). Furthermore miR-124-1 directly targets Bace1 and knowing the specific cell-types having decreased expression of miR-124-1 in an Alzheimer's Disease mouse model can allow for future studies to artificially increase miR-124-1 expression within these cell-types to reduce Bace1 expression (16, 17). Along with the pri-miRNAs known to be associated with Alzheimer's Disease, there are also other pri-miRNAs elucidated through this study which have not been studied. Further

study of these newly illuminated pri-miRNAs and targets of the mature form may reveal further regulation implicated in Alzheimer's Disease pathology.

Through use of primiReference in single-nucleus datasets, researchers will be able to characterize the understudied small regulatory RNAs involved in different conditions. For example, single-nucleus sequencing data of a knock-out of a transcription factor compared to wild-type may illuminate involvement in transcription of pri-miRNAs which target genes important in many pathways. Furthermore, use of this single-nucleus sequencing reference may reveal therapeutic targets for different diseases of the brain through comparisons of diseased to wild-type brain regions. Therefore, primiReference will be a useful, valuable, and impactful tool for researchers conducting research using single-nucleus datasets.

pri-miRNA Expression May Contribute to SVZ Dysfunction with Age

Cell-types within the SVZ rely on cell-cell communication to relay signals leading to NSC pool maintenance, differentiation, and neuroblast migration(18-26). During the aging process, there is global dysfunction leading to a reduction in NSC number and proliferation (27-29). Although some miRNAs effecting neurogenesis have also been implicated in the aging process, there has not been a global study of miRNA expression within each cell-type of the SVZ with age. Using primiReference, astrocytes were shown to have differential expression of pri-miRNAs with target genes enriched in the signaling pathways regulating pluripotency of stem cells, FoxO signaling, and Wnt signaling. In the signaling regulating pluripotency of stem cells pathway, IGF-1 is predicted to be targeted by miR-137, and the primary miRNA pri-miR-137 is increasing in expression with age in astrocytes. This is interesting since low IGF-1 signaling results in increased FoxO activation within the nucleus of target cells. In the case of NSCs, FoxO activation results in a quiescent state (30). With age, NSCs are known to become more quiescent, and this results in a decline in neurogenesis (31). Furthermore, astrocytes have

differentially expressed pri-miRNAs predicted to target Robo/Slit transcripts, which impacts chain migration of neuroblasts to the OB (32).

In addition to astrocytes, ependymal cells, microglia, and vascular endothelium were shown to have differentially expressed pri-miRNAs with enrichment of target genes in axon guidance, Wnt, and FoxO signaling. The net effect of these pri-miRNAs changing with age within these cell-types remains unknown. The impact of these changes on interacting cell-types additionally remains unknown. Not only are these miRNAs, potentially, impacting the signaling mechanisms from within the cell, but miRNAs are known to be packaged into vesicles and taken up by other cell types. Therefore, it is of interest which mature forms of pri-miRNAs are packaged into extracellular vesicles from astrocytes, ependymal cells, and microglia, and can then lead to differences in gene expression within the NSC cell type.

Within the actual NSCs and neural progenitors themselves, the impact of extracellular vesicles has been studied. miR-21a, for example, has been shown to be packaged into exosomes and sent to neighboring neural progenitors to stimulate neurogenesis (33). Within our dataset, pri-miR-21a is decreased among all cell-types. Since the report of miR-21a increasing neurogenesis was conducted *in vitro*, it is, perhaps, possible for miR-21a to be packaged and sent in exosomes from other cell-types in the SVZ as well to dictate neurogenesis. This reduction in pri-miR-21a, universally, in the SVZ with age may have far-reaching implications.

In addition to miR-21a, other miRNAs are validated to target genes involved in neurogenesis or oligodendrogenesis. miR-490, for example, decreases Hdac2 expression, and its expression is increased with age in NSCs (34). Hdac2 is required for proper differentiation of progenitors (35). Furthermore, pri-miR-219a-2, which is decreased in expression in OPCs and MFOLs with age, has been proven to increase oligodendrogenesis and remyelination (8, 9, 12). Since it is decreased in expression with age, it is likely that oligodendrogenesis and myelination are disrupted.

Together, this data suggests pri-miRNAs contribute to the dysfunction reported in age-associated decline of the SVZ. The extent to which these pri-miRNAs contribute to the dysfunction, however, remains unknown and will be the topic of future studies. miRNAs act to fine-tune the expression of their target genes and only reduce the expression of target mRNA. Therefore, functional studies of NSC survival, differentiation, and self-renewal will need to be conducted to determine if the changes in miRNA expression greatly contribute to the decline of NSC function. In addition to understanding functional aspects of miRNA differences in the SVZ, it is important to validate the predicted targets of the mature form of many pri-miRNAs elucidated in this study. Further complicating this data, it is unknown which miRNAs are ultimately only expressed within the cell-type or which are ultimately processed and packaged into extracellular vesicles and targeted towards NSCs. Although this study has led to a significant number of new questions, this study does lay a roadmap for understanding the role and contribution of pri-miRNAs to SVZ dysfunction, and this study shows a clear contribution of pri-miRNAs to the dysfunction based on validated data of target genes in literature. It is suggested from this transcriptomic data that there is a reduction in neurogenesis and impairment in neuroblast migration, however.

Developing primiReference for More Mouse Tissue Types and Human

The greatest limitation of primiReference currently is that it is only created and validated for use in mouse brain tissue. Since primiReference was created by overlapping publicly available data of pri-miRNA sequences from 6 cell lines with our single-nucleus SVZ data, there would need to be overlap with single-nucleus datasets from other tissue types within the mouse to create an accurate mapping of pri-miRNA sequences within those tissues. As is seen with the 6 cell lines, different cell-types have slight variations in pri-miRNA length (36). Furthermore, some tissues have tissue-specific miRNA expression, such as the liver (37).

By updating priReference for all tissue-types and for human as well, the differential expression of pri-miRNAs can be elucidated from innumerable conditions. Since single-cell technology has yet to include chemistry for small RNA detection, which necessitates different chemistry than regular sequencing at baseline, detection of primary miRNA transcripts is a step forward in determining regulatory RNA contribution towards gene regulation. priReference illuminates the often-forgotten transcriptional differences of regulatory RNAs for the future study of the impacts of their mature form on gene expression. Through priReference, another layer of transcriptomics may now be studied.

Discerning the Impact of Differentially Expressed pri-miRNAs on Age-Associated SVZ Dysfunction

Through single-nucleus sequencing data in this study, the differences in transcription of pri-miRNAs with age in SVZ cell-types was documented. The functional and mechanistic impact of these differences, however, must be determined as a next step. First, studies will need to be conducted to determine which predicted target genes are in fact directly targeted by the mature form of pri-miRNAs differentially expressed with age within each pathway. Once validated targets are confirmed, it will be important to study whether cell-types such as astrocytes, ependymal cells, or microglia have pri-miRNA expression that ultimately results in a change in the expression of signaling proteins involved in the development and differentiation pathways or if the mature miRNAs themselves are packaged and targeted to the NSCs for regulation within. It is interesting to consider that the pri-miRNA expression unearthed in this study within each cell-type may not be impactful on the cell-type itself but on a neighboring cell, rather. Therefore, if it is found, in future studies, that pri-miRNA transcription ultimately results in packaging of mature miRNA into exosomes, studies will need to be conducted to determine whether these exosomes are targeted to NSCs and the functional impact on NSC viability differentiation, and self-renewal discerned.

Tsix Expression is Increased in the Cell-Types of the Aging Female SVZ

When comparing aged to young female cell-types of the SVZ, every cell-type of the SVZ had a statistically significant increase in Tsix expression. Tsix antagonizes XIST expression, which prevents repression of both X chromosomes instead of just one (38). Tsix and Xist have coordinated transcription whereby the levels of Xist reach a threshold level and then expression of Tsix reach equilibrium thereafter under normal conditions (39). Furthermore, the expression of these two long non-coding RNAs (lnc-RNAs) leads to different chromatin organization structures (39, 40). Therefore, it is possible that an increase in Tsix expression may interfere with dosage of the X chromosome with age, may disrupt chromatin organization with age in the female cell-types of the SVZ, or both.

Since epigenetic alterations are a component of the hallmarks of aging, it is thus exciting to contemplate the consequences of increased Tsix expression in aged female SVZ cell-types. Recently, Tsix has been associated with expression of frailty genes in humans (41). Perhaps increased Tsix expression, under these conditions, has the same detrimental effects. Although gene expression was similar among female and male cell-types with age, the increased Tsix expression may help explain the differences in expression of some genes on the X chromosome. The similarity among gene expression may also be due to general variability of gene expression with age, however. Therefore, experiments will need to be conducted to determine if increased expression of Tsix impacts overall chromatin organization, if dosage of genes on the X chromosome are changed in expression, and if any of these possible changes cause differences in cell-type specific function such as NSC self-renewal capacity or ependymal cell filtration of CSF. Together, these experiments will help to determine if females have unique changes in gene dosage guiding aging in the SVZ and perhaps contributing to enhanced dysfunction or function with age.

Wnt Signaling May Be Differentially Regulated in the Female and Male SVZ with Age

Through Ingenuity Pathway Analysis (Qiagen IPA), the transcription factor Tcf7l2 was highlighted as an upstream regulator to many genes differentially expressed within every cell-type of the male SVZ with age. Tcf7l2 is involved in Wnt/ β -catenin signaling as well as regulation of insulin signaling genes, regulation of oligodendrogenesis, and more (42-44). Interestingly, Tcf7l2 is decreased in expression in the male OPC cluster with age, and studies have shown that augmentation of Tcf7l2 leads to decreased Bmp4 transcription and secretion leading to decreased OPC differentiation (42). Therefore, it is possible that this decrease in the OPC cluster may be affecting OPC differentiation, although BMP4 was not shown to be differentially expressed. Single-nucleus sequencing data is, at times, incapable of detecting low-expressed genes or particular transcripts, so further studies are in need to determine if the decrease in Tcf7l2 in OPCs is functionally meaningful (2). Therefore, functional studies on OPC differentiation, with decreased Tcf7l2, should be conducted to determine if Wnt signaling and differentiation are impaired.

Out of the 37 targets of Tcf7l2 present in every cell-type differentially expressed with age, 36 of the targets decreased in expression in male SVZ cell-types. Therefore, Tcf7l2 is predicted to be decreased in activation as a transcription factor for these targets. Since Wnt/ β -catenin signaling is overrepresented in some aged male SVZ cell-types compared to young male SVZ cell-types, it would be interesting to determine if canonical Wnt signaling is decreased or if calcium-dependent Wnt signaling is inhibiting Tcf7l2 dependent transcription (45-47). Transcriptional decrease in Daam1 is seen in every cell-type as well, and it will be interesting to explore if this leads to decreases in the non-canonical/planar cell polarity Wnt pathway signal transduction (**Figure 1**). Therefore, studies on functional outcomes of Daam1 inhibition should be conducted in the SVZ cell-types *in vitro* and in the SVZ as a whole *in vivo* to determine if cytoskeletal structure is affected or if NSC quiescence or neuroblast migration is affected.

On the other hand, Tcf7l2 transcription is decreased in female SVZ cell-types with age. It will need to be determined if Tcf7l2 activation is additionally decreased. If canonical Wnt signaling is decreased in the female SVZ, neurogenesis will likely decline (**Figure 1**). This will need to be explored in both NSC and progenitor cell-types as well as cell-types secreting Wnt signaling molecules such as astrocytes and ependymal cells. Tcf7l2 decreased activation will have different outcomes within each cell-type such as a decreased secretion of Wnt signaling molecules affecting surrounding cell-types.

It will be interesting to determine if the female and male SVZ have sex-specific differences in Wnt signaling with age since that can help determine differential mechanisms of survival or decline. For example, disruptions to planar cell polarity Wnt signaling may disrupt migration of neuroblasts or maturation of new neurons more in the male than female with age (45) (**Figure 1**). Therefore, once pathway regulation is disentangled, functional assays as such as neurosphere formation, viability, and differentiation assays will need to be used to determine the ultimate outcome of these changes within each sex.

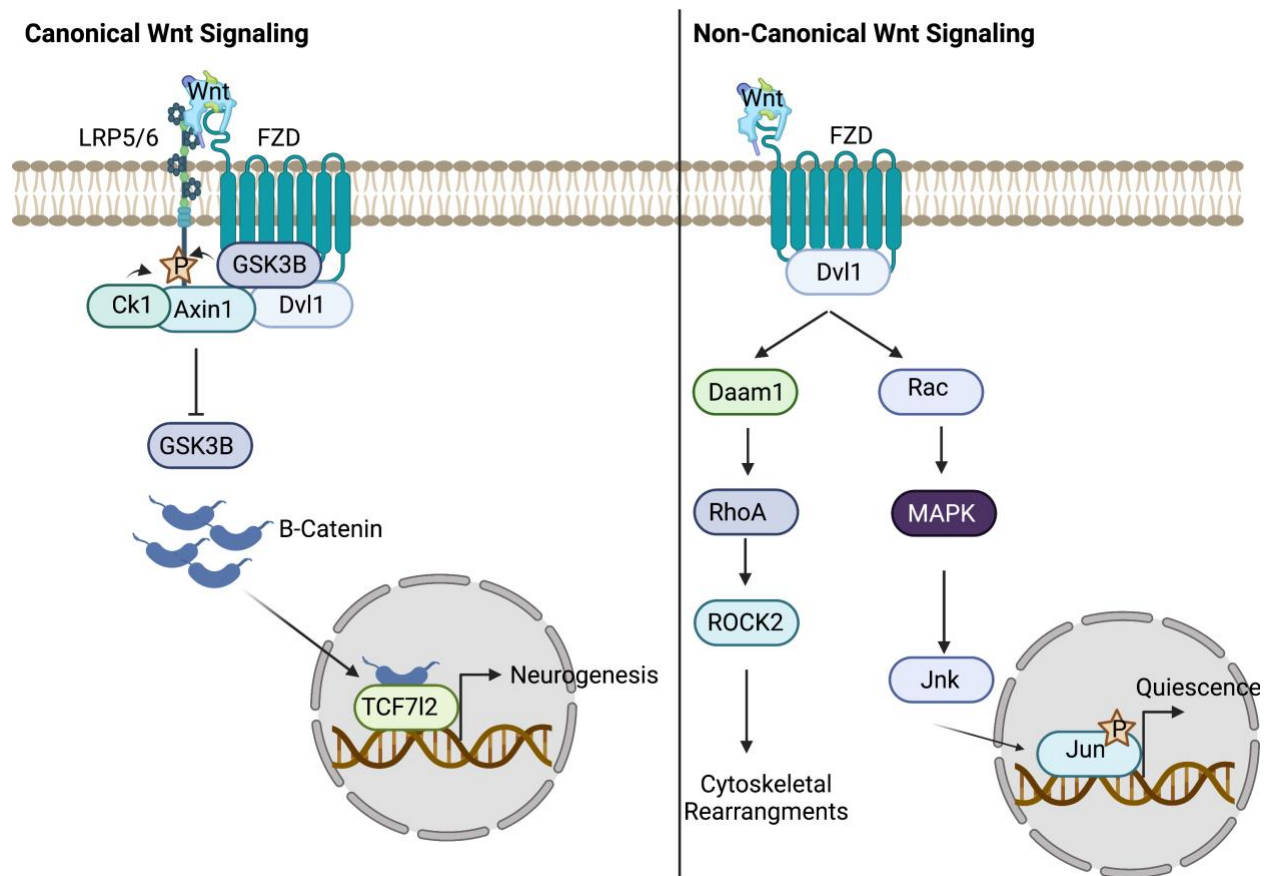


Figure 1: Differences in canonical and non-canonical Wnt signaling. Wnt signaling through LRP5/6 and Fzd leads to inhibition of GSK3B and translocation of β -catenin to the nucleus to activate Tcf7I2 transcription of genes leading to neurogenesis. Non-canonical or Planar Cell Polarity (PCP) Wnt signaling activates Daam1 to signal through RhoA and ROCK2 leading to cytoskeletal rearrangement or through Rac and MAPK pathways to activate Jun transcription of target genes leading to quiescence. Tcf7I2 is decreased in transcription in female cells while it is predicted to be inhibited in male cell types with age. Daam1 is transcriptionally decreased in male cell-types with age.

Female and Male SVZ Cell-Types Have Differential Expression of Calcium Signaling Genes with Age and Between the Sexes

Calcium signaling is hugely important for development and differentiation of the SVZ. With age, the female SVZ has a decrease in a subunit of the Cav1.2 L-type voltage gated ion channel transcribed by *Cacna1c*. Additionally, the female SVZ cell-types have decreased transcription of the *ErbB4* receptor and transcription factor *Creb5*. *ErbB4* transcription is regulated by estrogen, and the protein is involved in neuroblast migration (48-50). Meanwhile, *Creb* is responsible for initiating transcription of genes with cAMP response elements (CREs) and has additional roles in estrogen signaling and transcription of target genes (51). In the SVZ, CREB aids in NSC survival and expression of neuronal genes during differentiation (52).

On the other hand, the male SVZ cell-types have increased expression of *Cacna1c*. Both sexes have increased expression of *Adcy1*, *Calm1*, *Camk2a*, and *Camk2n1* with age, however. *Adcy1* catalyzes cAMP production, specifically in the brain (53). *Calm1* is a calcium sensor, that can be activated by influx of calcium via *CaV1.2*, which can activate *Camk2a* (*CamkIIa*) leading to downstream calcium-dependent signaling cascades (54-56). *Camk2n1* is the inhibitor of *Camk2a* activation (57, 58). When comparing differences in this expression between the sexes, *Cacna1c* is increased in expression in the young female SVZ cell-types compared to the male while *Calm-1* is decreased in expression in the young female SVZ cell-types compared to the male. Conversely, in the old-aged comparison, *Cacna1c* is decreased in expression in the female cell-types while *Calm1* is increased in expression in the female cell-types. This flip-flop of gene expression is fascinating since calcium signaling is crucial to the processes of differentiation and migration in NSCs, and there are both age and sex-dependent differences.

The difference in *Cacna1c* expression with age can be explained since *Cacna1c* contains an estrogen response element, and estrogen expression decreases with age in females. Additionally, some genes were increased in expression within young female cell-types compared to male cell-types, but the expression was unchanged in the old comparison. *Chrm2*, for example, is regulated by an ERE as well, and the expression was increased in young yet

unchanged in old (59). Therefore, estrogen may play a large role in regulating calcium signaling, and likely more pathways, with age in the female SVZ. Declining estrogen, perhaps, is responsible for the similarity in gene expression between old female SVZ cell-types and old male SVZ cell-types as well. Consequently, changes in expression of genes with EREs must be investigated to determine if decreased estrogen within the female with age is responsible for global changes in gene expression. Furthermore, experiments focused on chromatin binding of Creb will need to be determined to characterize whether these changes are due to Creb binding to cAMP response elements (CREs) or EREs, since some genes contain both (**Figure 2**). Once the effects of estrogen on gene transcription are determined, in an age-associated manner, the ultimate effect of these transcriptional changes will need to be elucidated to determine if these changes are responsible for SVZ dysfunction with age in the female. As a further experiment, it will need to be determined if these changes in gene expression are responsible for the similarity in gene expression between aged female and male cell-types. In this study, the estrous cycles were not synchronized, so a repeated study in which estrous cycles are synchronized will help to better determine how estrogen levels are affecting gene expression.

Since *Cacna1c* is decreased in expression, yet the downstream effectors are increasing in expression in the aged female SVZ, the question becomes is calcium entry inhibited (**Figure 2**)? To answer this question, further studies will need to be conducted by imaging calcium flux within aged SVZ tissue. Furthermore, calcium entry via *Cacna1c* is linked to MAPK signaling, which is vital to neurogenesis (55, 60). Therefore, if calcium entry is, in fact, impaired then MAPK signal transduction and neurogenesis are perhaps decreased. Transcriptionally, we would not see any difference in our data since these are regulated through phosphorylation events, although downstream target genes of MAPK signaling should be further explored.

This may help to explain the overrepresentation for MAPK signaling genes in male the SVZ with age and absence in the female SVZ with age. The overrepresentation of these genes, however, are split between upregulated and downregulated with age. Those upregulated with

age include calcium receptors while those downregulated with age include Tgfb β 1 (TGF- β receptor), Pak1 (p21 activated Rac1 kinase), Mapt (Tau). Although these are generally downregulated, there is cell-type specificity regarding which genes in the MAPK pathway are upregulated and downregulated. Therefore, the two most important experimental questions to answer will be is transcriptional regulation of calcium and MAPK genes related to protein content? And does the decrease or increase in transcription lead to differences in signal transduction ultimately?

Since calcium signaling can activate transcription factors such as Creb and NFAT while also regulating gene expression/repression through DREAM, it is imperative to determine the ultimate effect differential expression of Cacna1c and Calm1 have with age and between the sexes (**Figure 2**). These changes in transcription factor activation ultimately control everything from NSC proliferation to lineage fate determination, neuroblast migration, and neuronal maturation. It will be interesting to determine if cell-type specific differences in Cacna1c expression with age ultimately lead to differences involved in age-associated SVZ dysfunction (61-64). Thus, activation states of Calm1 and downstream effectors such as CamkIIa and Camk2n1 must be determined to see the ultimate outcome of the signaling cascade (**Figure 2**) Without knowing the activation states of these proteins, it is impossible to determine the exact outcome of these transcriptional differences, unfortunately, but these discoveries leave the door open to far more studies of this complicated pathway and the implications of differential expression of calcium receptors in an age and sex dependent manner.

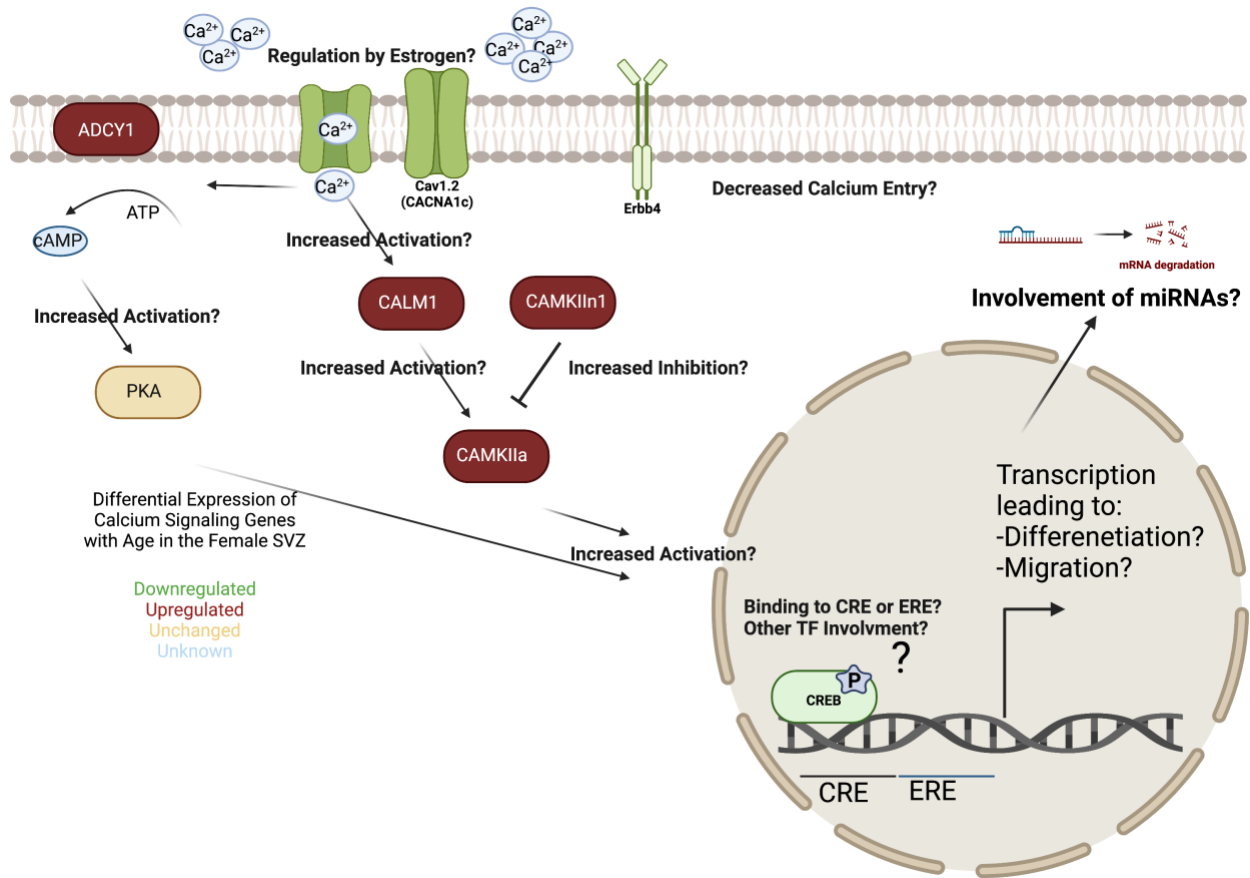


Figure 2: A Model for Differences in Calcium Signaling with Age in the Female SVZ with

Remaining Questions: This model shows genes increased in expression with age in the female SVZ in the calcium signaling pathway such as ADCY1, CALM1, CAMKIIa, and CAMKIIin1 as well as genes decreased in expression with age such as CACNA1C, ERBB4, and CREB5. The binding elements shown are cAMP Response Elements (CRE) and Estrogen Response Elements (EREs). This model includes questions that need to be answered to further understand the impact of transcriptional gene expression on pathway transduction and eventual impact on SVZ cell-types and function.

Intersection of Wnt and Calcium Signaling Differences in Male and Female Aged SVZ

Cell-Types

In the female cell-types, Rorb (Ror2) is decreased in expression in age in most cell-types with age, while Rora (Ror1) is increased in expression in most cell-types in the male SVZ with age. This is very fascinating since non-canonical Wnt signaling can transduce through Rora/b leading to calmodulin and CamkIIa activation (46, 65). Signaling through Rorb can activate the planar cell polarity pathway of Wnt signaling thus leading to increased differentiation and migration(46, 65, 66). On the other hand, since we see decreased Rorb and decreased Tcf7l2 in female cell-types with age, there is a possible decrease in both Wnt/Ca²⁺ and canonical Wnt signaling (**Figure 3**). Decreases in Rora and Rorb have been shown to decrease neural progenitor cell division and thus neuroblast formation (66). Therefore, this may add another layer of complexity to be further explored whereby Wnt and calcium signaling may be differentially regulated between the female and male SVZ with age (**Figure 3**). It is possible that the male SVZ shifts to Wnt/Ca²⁺ signaling with age since the signaling cascade blocks Tcf7l2-dependent transcription (66, 67). With the transcriptional data on hand, it appears as though females have an overall downregulation of canonical Wnt signaling and perhaps even Wnt/Ca²⁺ signaling as well. More studies must be conducted to determine protein levels and activation states before the effects of these transcriptional differences between females and males with age can be conclusively determined. These studies must be conducted in a cell-type specific manner to determine if the manufacturing and signaling of Wnt proteins are affected or if the downstream transcriptional changes are transduced within a cell-type. Therefore, the difference in transcription of NFAT, c-Jun, and Tcf7l2 target genes must be elucidated to determine if there is a clear preference for a particular Wnt signaling pathway. The outcome of these changes should then be assessed using co-culture and functional assays for neural stem cell self-renewal, differentiation, and viability.

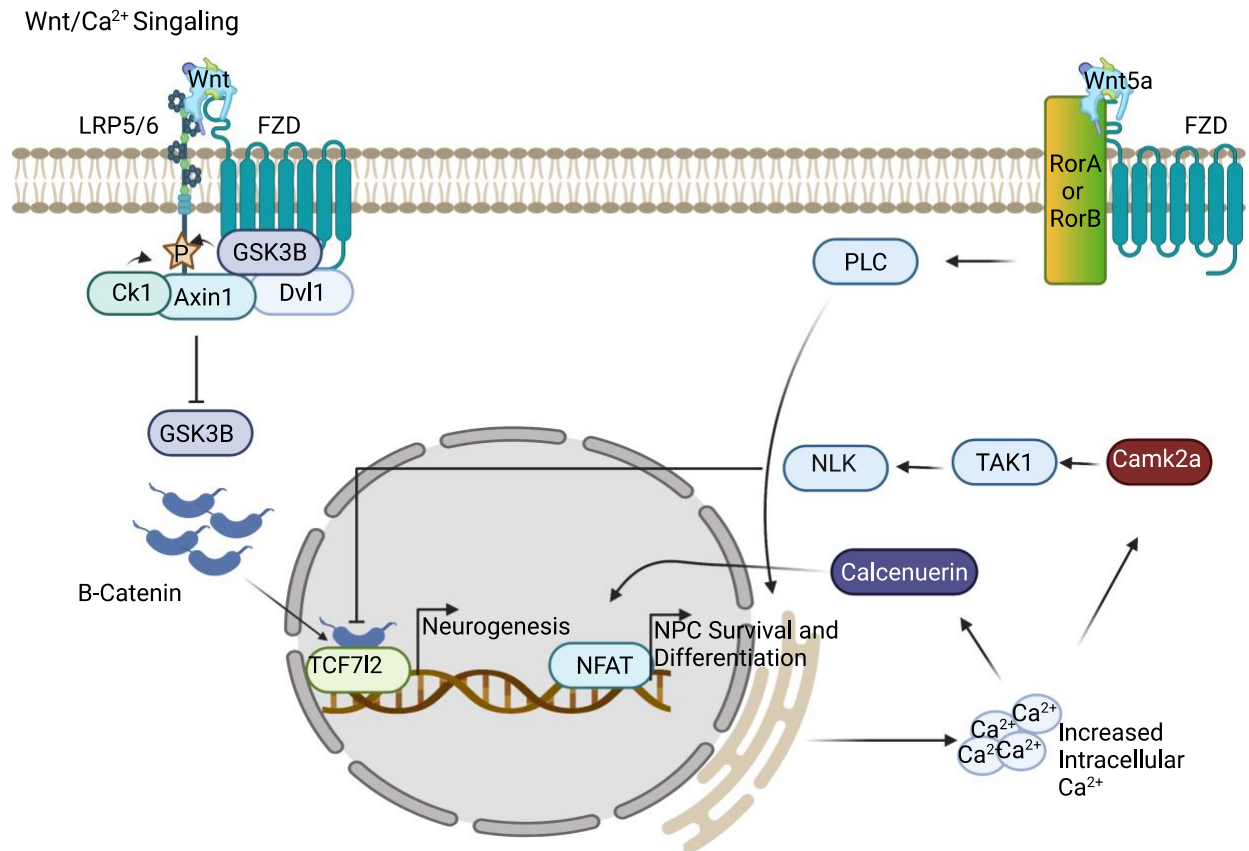


Figure 3: Wnt/ Ca^{2+} Signaling. In this figure Wnt is signaling through the canonical pathway shown on the right and through β -catenin to induce Tcf7I2 transcription. In females, Tcf7I2 is reduced in expression with age while in males it appears to be reduced in activity based on a reduction in target gene expression. Additionally, Wnt5a can signal through Rora/Rorb to increase intracellular calcium levels. Rora is increased in males with age while Rorb is decreased in females with age. Signaling through calcium can either induce the Camk2a pathway which blocks Tcf7I2 transcriptional activation of target genes or through the calcineurin pathway to activate NFAT transcription of genes.

pri-miRNA Expression is More Similar in Aged SVZ Cell-Types of the Female and Male Brain

When comparing young female SVZ cell types to young male SVZ cell-types, there were far more differences in pri-miRNA expression than when comparing the aged cell-types in a sex specific manner. In the young sex-specific comparison, the pri-miRNAs were predicted to target genes mostly within the development and differentiation pathways such as the Wnt, FoxO, MAPK, and signaling regulating the pluripotency of stem cells pathway. The pri-miRNAs with enrichment of predicted target genes in these pathways were mostly the astrocyte, TAP, OPC, and vascular endothelium clusters. Therefore, it is paramount to determine which targets are valid and then if the mature form of these pri-miRNAs are targeting the genes within the cell-type or if they are packaged into vesicles and targeted towards other cell-types. Furthermore, it must then be determined what the contribution of these pri-miRNAs is on SVZ function and how that differs between the sexes in the young SVZ.

In the old age group, it is interesting that the predicted target genes of pri-miRNAs differentially expressed between female and male SVZ cell-types are mostly enriched in glutamatergic synapse and calcium signaling pathways. Genes belonging to both pathways are involved in calcium signaling and ultimately regulation of NSC proliferation, differentiation, and migration (63). Therefore, there may be a “one-two punch” mechanism guiding expression of genes in the calcium signaling pathway between the sexes. This mechanism would involve differences in both transcriptional regulation and then regulation of gene expression via miRNA regulation thus causing increasingly different protein profiles and ultimately signaling outcomes.

Conclusions: pri-miRNA data can be elucidated from single-nucleus sequencing using primiReference, and it shows pri-miRNAs likely contribute to SVZ decline. Additionally, pri-miRNA and gene expression are drastically different between aged and young SVZ cell-types within both the female and male SVZ. With age, however, gene expression becomes more similar between the female and male SVZ cell-types. Ultimately, there appears to be transcriptional differences in calcium signaling and Wnt signaling genes, as well as the pri-

miRNAs that are predicted to target some of these genes, that may be regulated, in part, by differences in sex-steroid levels with age.

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Appendix

ORIGINAL PAPER

WILEY Aging Cell



The mir-465 family is upregulated with age and attenuates growth hormone signaling in mouse liver

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Abstract

We analyzed the small RNA transcriptome from 5-month-old, 24-month-old, and 36-month-old mouse liver and found 56 miRNAs that changed their expression profile with age. Among these is a cluster of 18 miRNAs that are upregulated between 50- and 1,000-fold at 24 and 36 months of age. This cluster is located in a 60-kb region of the X-chromosome that is devoid of other coding sequences and is part of a lamin-associated domain. Potential targets of the miRNAs in the cluster suggest they may regulate several pathways altered in aging, including the PI3K-Akt pathway. Total transcriptome analyses indicate that expression of several potential genes in the PI3K-Akt pathway that may be targeted by the mir-465 family (mmu-mir-465a, mmu-mir-465b, and mmu-mir-465c) is downregulated with age. Transfection of the liver cell line AML12 with mir-465 family members leads to a reduction of three of these potential targets at the mRNA level: a 40% reduction of the growth hormone receptor (GHR), and a 25% reduction in Kitl and PPP2R3C. Further investigation of the GHR 3'UTR revealed that the mir-465 family directly targets the GHR mRNA. Cells transfected with mir-465 showed a reduction of JAK2 and STAT5 phosphorylation upon growth hormone (GH) stimulation, resulting in a reduction in insulin-like growth factor 1 (IGF-1) and IGF-1-binding protein 3 expression. With age, GH signaling falls and there is a reduction in circulating IGF-1. Our data suggest that an increase in expression of the mir-465 family with age may contribute to the reduction in the GH signaling.

KEYWORDS

aging, growth hormone receptor, growth hormone signaling, liver, miRNAs, mouse

1 | INTRODUCTION

Alterations in growth hormone (GH) signaling affect healthspan and lifespan in a wide range of organisms ranging from invertebrates to humans (Bartke, List, & Kopchick, 2016; Barzilai, Huffman, Muzumdar, & Bartke, 2012). The GH signaling pathway plays a key role in metabolic regulation through activation of multiple signaling pathways including the IGF-1 signaling pathway, the MAP kinase

pathway, and the PI3K-Akt pathway. GH signaling is thought to influence lifespan through its action on the somatotrophic axis (Bartke et al., 2016). GH is synthesized in the anterior pituitary gland and secreted into the bloodstream upon hormonal stimulation where it acts primarily on the liver through binding to the GH receptor (GHR). Activation of the GHR by GH binding leads to the phosphorylation of Janus-family tyrosine kinase 2 (JAK2), which subsequently

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phosphorylates several transcription factors resulting in their activation, including the STAT5b transcription factor that stimulates insulin-like growth factor 1 (IGF-1) expression (Alvarez-Nava & Lanes, 2017). IGF-1 is released into the bloodstream as a complex with IGF-binding protein 3 (IGFBP3) and IGF acid-labile subunit (IGFALS) where it targets numerous cell types to regulate metabolism, growth, and development.

Many studies have looked at the effects of a lifelong, global reduction in growth hormone signaling by knocking out various components of the GH signaling pathway. Interestingly, the majority of these studies resulted in healthspan and lifespan extension (reviewed in Brown-Borg, 2015). These results appear to be applicable to humans as centenarians have higher frequencies IGF1R gene polymorphisms that result in altered IGF-1 signaling (Suh et al., 2008). In addition, individuals with Laron dwarfism, which results from a mutation in the growth hormone receptor gene, have decreased incidence of diabetes and cancer; however, they do not have an extended lifespan (Guevara-Aguirre et al., 2011). Taken together, these results suggest that a reduction in GH signaling has a beneficial effect on healthspan and lifespan.

However, the role of GH signaling in determining healthspan and lifespan does not appear to be that clear-cut. GH signaling normally declines with age and this reduction has been established as a risk factor for developing diabetes, cardiovascular disease, sarcopenia, osteoporosis, and frailty (Bartke et al., 2016; Barzilai et al., 2012), and sustained elevated GH signaling increases the risk for some cancers (Barzilai et al., 2012). Delivering GH to GH-deficient mice during the juvenile period diminishes the lifespan-extending effects, whereas the administration of GH during adulthood does not affect lifespan (Panici et al., 2010; Sun et al., 2017). These results suggest that there is a narrow window in preadulthood when a reduction in growth hormone signaling can influence lifespan. In addition, liver-specific reduction in IGF-1 in middle-aged mice had negative consequences including liver inflammation, accelerated bone loss, oxidative stress in various tissues, and increased hepatic tumors leading to a reduction in healthspan (Gong et al., 2014). These effects are thought to be caused by an increase in circulating GH, resulting from interference with the normal negative feedback loop of IGF-1 on GH release (List et al., 2014). Collectively, these studies suggest that GH signaling must occur in an optimized manner to promote healthy aging, and that these interactions are more complex than previously believed.

The natural decline in GH signaling with age is associated with the onset of age-associated diseases and pathologies. Therefore, the mechanisms that result in these natural changes in GH expression during our lifespans are of significant interest but remain poorly understood. Gene expression is controlled at multiple points from regulation of transcription by epigenetic features to modulation of mRNA levels by small, noncoding RNAs. MicroRNAs (miRNAs) are a class of small, noncoding RNAs that regulate mRNA expression at the posttranscriptional level. Mature miRNAs target specific mRNAs through recognition of a sequence in the 3'UTR that is partially complementary to nucleotides 2–7 of the miRNA (the seed sequence), targeting the mRNA for degradation or inhibition of translation.

Here, we report that a cluster of miRNAs located in a highly heterochromatic region of the X-chromosome is derepressed with age in mouse liver. This cluster includes members of the mir-465 family, which we show downregulate GHR expression. A reduction in GHR expression will lead to alterations in growth hormone signaling that ultimately could affect both healthspan and lifespan.

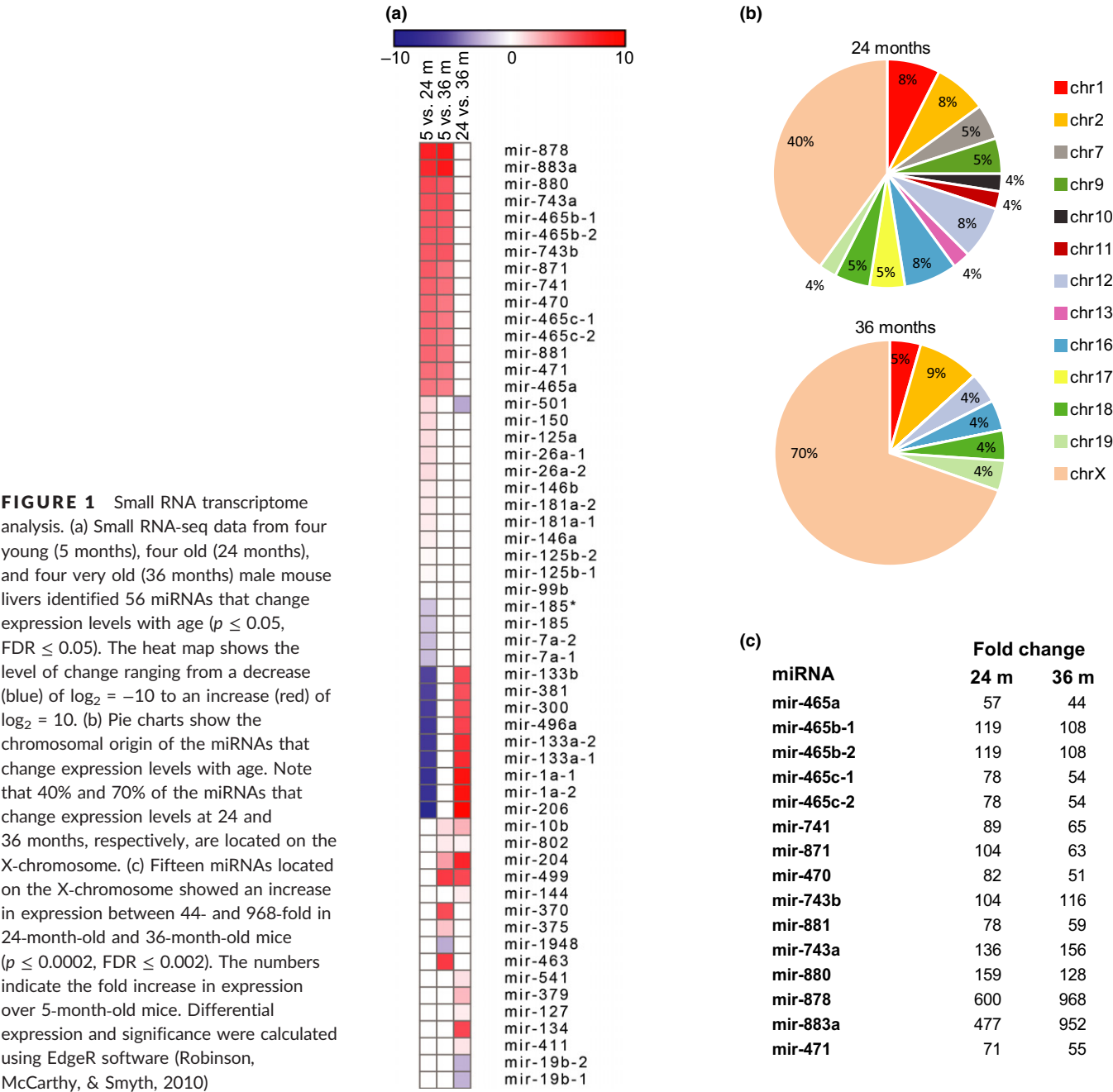
2 | RESULTS

2.1 | Deep sequence analysis of the small RNA fraction in young and old mouse liver

To investigate changes in miRNA gene expression that occur with normal aging, we performed deep sequence analysis of the small RNA fraction (<200nt) isolated from male mouse liver at different times during the mouse lifespan. C57BL/6 mice reach adulthood by 4 months of age and have a median lifespan of 27 months and a maximum lifespan of about 39.5 months (The Jackson Laboratory, 2018). Therefore, to encompass the full lifespan, we sequenced the small RNA fraction from young (5 month), old (24 month), and very old (36 month) mice. We found 56 miRNAs that changed expression levels with age (Figure 1a). The majority showed increased expression, with only 14 miRNAs decreasing in expression at 24 or 36 months of age. Further examination of the data showed that approximately 40% and 70% of the miRNAs that show significant changes in expression at 24 and 36 months of age, respectively, are located on the X-chromosome (Figure 1b). We identified a cluster of 15 miRNAs on the X-chromosome that are significantly upregulated at both ages (Figure 1c). Members of this cluster are expressed at very low levels in young animals and are upregulated between 50- and 600-fold by 24 months of age. Their expression remains elevated (compared to 5-month liver) at 36 months indicating that the upregulation of this miRNA cluster is not transient or reversed with advanced age.

To determine the dynamics of this increase in expression of the miRNA cluster, we analyzed expression of the miRNAs in liver by quantitative PCR (qPCR) across the mouse lifespan. It was previously shown that these miRNAs are not expressed at significant levels outside of the testes in young mice (Landgraf et al., 2007; Ro, Park, Young, Sanders, & Yan, 2007). We found that expression of most of the miRNAs remains low through 18 months of age, confirming this previous finding. However, mir-871 and mir-471 show increased expression beginning at 12 months of age, and mir-465b begins to show increased expression by 18 months of age (Figure 2a). Between 18 and 24 months of age, expression of other members of this miRNA cluster goes up significantly (Figure 2b). There was significant variation between individual animals, but overall they all showed elevated levels of expression of these miRNAs at 24 and 36 months of age (Supporting Information Figure S1).

The age-associated upregulation of this locus was not limited to liver, as increased expression also occurred in skeletal muscle, brain, and senescent-cultured mouse tail fibroblasts, although to a lesser extent (Supporting Information Figure S2). In skeletal muscle,



expression of these miRNAs increased between fourfold and ninefold at 24 months of age, followed by a decrease to around twofold by 36 months (compared to 5 months). In brain, we did not see an increase in expression at 24 months, and only a subset of these elements showed a modest increase in expression (1.5-fold) by 36 months of age. Senescent mouse tail fibroblasts showed between 1.7- and threefold induction of these miRNAs. These results suggest that the large increase in expression of these miRNAs with age is not universal and may be specific to the liver.

The 15 upregulated miRNAs map to a 60-kb region of the X-chromosome (Figure 3). Further analysis of this region revealed a total of 18 miRNAs present in the cluster. This region is devoid of other coding genes and is rich in repetitive elements, especially in

long interspersed nuclear elements (LINEs). There is an enrichment of laminB1-binding domains in this region in other mouse tissue types, suggesting that this region is part of a lamin-associated domain (Peric-Hupkes et al., 2010). Lamin-associated domains are typically comprised of heterochromatin that remains tightly repressed throughout the lifecycle of the cell. Our data showing increased expression of miRNAs in this region suggest that repression of this region may be lost with advancing age.

2.2 | Targets of the miRNAs in the X-cluster

The targets of the 18 miRNAs have not been well characterized in the literature. Our bioinformatic analyses of putative targets in

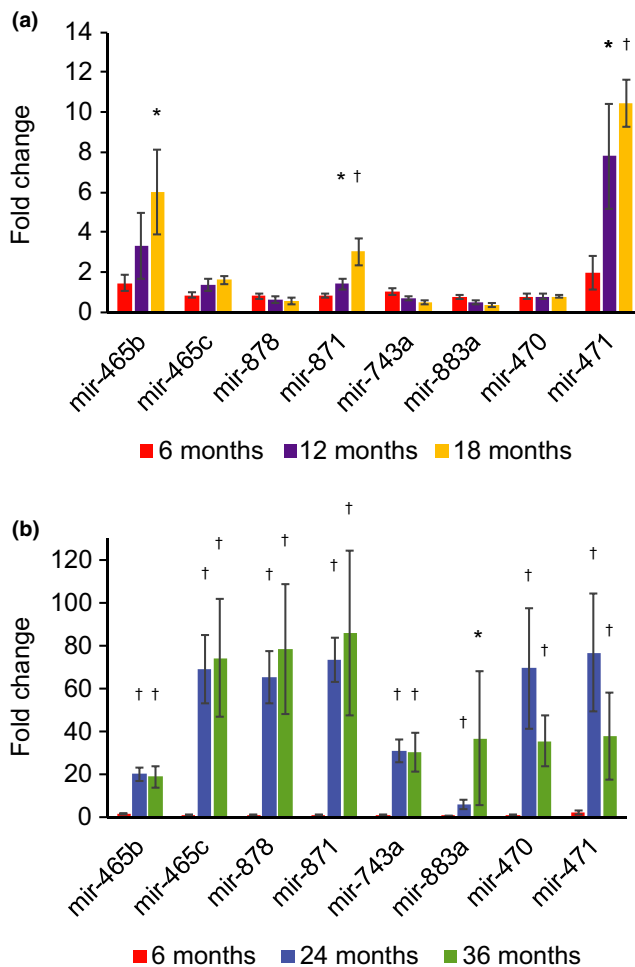


FIGURE 2 Expression of the X-chromosomal miRNA cluster with age. Expression of eight miRNAs in the X-chromosomal cluster was analyzed over the mouse lifespan at 6, 12, 18, 24, and 36 months of age by quantitative PCR. (a) Significant increases of 1.5-fold and 7.8-fold were seen in mir-871 and mir-471, respectively, by 12 months of age. Expression of mir-465b increased 3.3-fold at 12 months, but was not significant. Expression of these three miRNAs continued to increase significantly at 18 months of age, with mir-465b increasing sixfold, mir-871 increasing threefold, and mir-471 increasing 10.5-fold when compared to 6-month-old animals. (b) Expression of all eight miRNAs examined increased significantly at 24 and 36 months of age when compared to expression levels at 6 months of age. The increase in expression ranged from 5.8- to 76.9-fold at 24 months of age and from 18.8- to 86.1-fold at 36 months of age. Each bar represents the average expression level from three or four individual animals at that age performed in triplicate. Error bars represent standard errors. A representative experiment showing values for individual animals is shown in Supporting Information Figure S1. All p -values were calculated using two-tailed Student's t test. * $p < 0.05$, † $p < 0.01$ compared to 6-month-old animals

the 3'UTR of known mRNAs show that members of this miRNA cluster have the potential to influence several major pathways known to be altered in normal aging (Table 1). Four of the top 10 pathways predicted to be affected by expression of these miRNA include the PI3K-Akt, mTOR, MAPK, and Wnt signaling pathways. These pathways are highly intertwined and show aberrant

expression in several of the major diseases of aging, including cancer. We focused our initial investigations on the PI3K-Akt pathway since there are 69 mRNAs in this pathway that contain potential target sequences in their 3'UTR that may be recognized by at least one of 14 miRNAs in the cluster. Analysis of the liver transcriptome by RNA-seq revealed that 28 of the 64 predicted mRNA targets showed decreased expression levels in old (24 months) and very old (36 months) mouse liver when compared to young (5 months) mice. These targets included receptors, ligands, and transcription factors associated with PI3K-AKT signaling, indicating that attenuation of signaling by these miRNAs may occur at multiple points along the pathway. To verify that these mRNAs are reduced with age, we performed quantitative PCR (qPCR) on 5-month, 24-month, and 36-month mouse liver. We focused our investigation on the predicted mRNA targets of the mir-465 family, since nine of the 28 predicted mRNA targets in the PI3K-AKT pathway that are reduced with age in RNA-seq analyses are potential targets of this miRNA family. The mir-465 miRNA family is a subset of the upregulated X-chromosomal cluster and consists of five copies of the mir-465 genes in a 13-kb region of the X-chromosome (Figure 3). We validated that seven of the predicted targets in the PI3K-Akt pathway have reduced expression in aged mouse liver by qPCR (Figure 4a). These include GHR, Kit ligand (Kitl), protein phosphatase 2 regulatory subunit B", gamma (PPP2R3C), vascular endothelial growth factor A (Vegfa), guanine nucleotide-binding protein beta 1 (Gnb1), Kirsten rat sarcoma viral oncogene homolog (Kras), and the phosphatase and tensin homolog (PTEN). These results establish a connection between an age-associated decrease in expression of members of the PI3K-Akt pathway with an increase in expression of the mir-465 family that has the potential to regulate their mRNA stability.

To determine whether the predicted targets that are reduced with age are true targets of the mir-465 family, we transfected the mouse liver cell line AML12 (Wu, Merlino, & Fausto, 1994) with either a plasmid expressing all three mir-465 family members (pmir465abc) or with a mir-465c mimic. Expression of the mir-465 family was increased nearly 100-fold upon transfection with pmir465abc into AML12 cells (Supporting Information Figure S3). Transfection of the pmir465abc plasmid or the mir-465c mimic led to a 40% reduction of the GHR mRNA expression, and an approximate 25% reduction of the Kitl and PPP2R3C mRNA expression (Figure 4b). We did not see a significant reduction in expression of Vegfa, Gnb1, Kras, or PTEN mRNA, indicating that these may not be true targets of the mir-465 family, or that the mir-465 family may act by inhibiting translation of these target mRNAs instead of initiating degradation.

We focused our investigations on the GHR, since the liver is an important site of GH signaling and the GH signaling pathway is known to be involved in the regulation of normal aging. To determine whether expression of the mir-465 family leads to a reduction in GHR protein levels, we performed western blotting on AML12 cell extracts and found a 50% decrease in GHR protein levels in cells transfected with the mir-465 family (Figure 4c). These experiments further substantiate that the mir-465 family targets the GHR mRNA leading to a reduction in expression of the GHR protein.

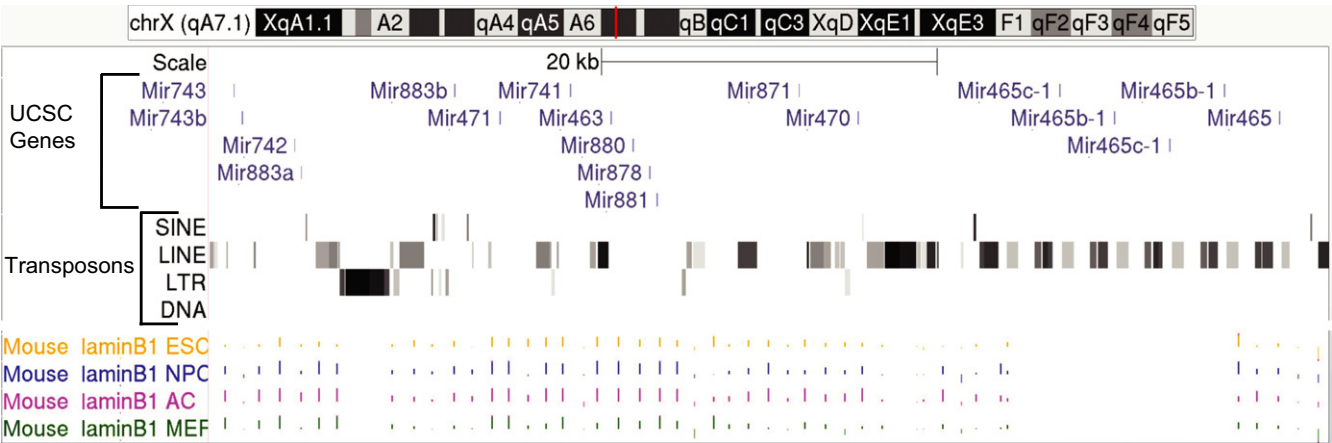


FIGURE 3 Genomic location of the X-chromosome miRNA cluster. The cluster of miRNAs is located in a 60-kb region of the X-chromosome (<http://genome.ucsc.edu/>; Kent et al., 2002). There are a total of 18 miRNA genes located in this region, which is devoid of other coding sequences and is rich in transposons, including short interspersed nuclear elements (SINE), long interspersed nuclear elements (LINE), long terminal repeats (LTR), and DNA transposons (DNA). This region is known to be part of a lamin B1-associated region in mouse embryonic stem cells (ESC), neural progenitor cells (NPC), astrocytes (AC), and embryonic fibroblasts (MEF; Peric-Hupkes et al., 2010), which indicates that it is part of a heterochromatic region of the genome. The red line indicates chromosomal location of the miRNA cluster

TABLE 1 Pathways predicted to be affected by miRNAs in the X-chromosomal cluster

KEGG pathway	p-Value	Genes ^a	miRNAs ^b	RNA-seq verified ^c
PI3K-AKT signaling pathway	3.02 x 10 ⁻¹⁰	69	14	28
mTOR signaling pathway	2.63 x 10 ⁻⁷	17	11	7
Axon guidance	2.93 x 10 ⁻⁷	30	11	ND
GABAergic synapse	8.26 x 10 ⁻⁷	17	7	ND
Prostate cancer	1.57 x 10 ⁻⁶	20	10	ND
MAPK signaling pathway	6.77 x 10 ⁻⁶	45	11	9
Dopaminergic synapse	6.91 x 10 ⁻⁵	26	10	ND
Glutamatergic synapse	9.25 x 10 ⁻⁵	22	8	ND
Wnt signaling pathway	9.47 x 10 ⁻⁵	29	9	12

Notes. ND: not determined.
Pathways in bold are known to be altered with age.
^aThe number of genes in the pathway that are predicted to be affected by at least one miRNA in the cluster. ^bThe number of miRNAs predicted to affect at least one gene in the pathway. ^cThe number of predicted genes shown to decrease with age by RNA-seq.

Further examination of the mir-465 target site in the GHR 3'UTR showed that this site is highly conserved across a wide range of mammalian species from shrews to humans (Supporting Information Figure S4), indicating that it may be important for regulating GHR expression and consequently GH signaling. To verify that the predicted target site of the mir-465 family is functional, we cloned the 3'UTR of the growth hormone receptor gene into the pmirGlo luminescent reporter plasmid. When the reporter plasmid was cotransfected with a mixture of miRNA mimics containing

all three mir-465 family members into HEK-293T cells, it led to a 50% reduction in luminescence signal (Figure 4d). Mutation of the target site in the GHR 3'UTR restored luminescence indicating that the predicted site in the GHR 3'UTR is an active site recognized by mir-465 family members. To determine whether differences exist between the activity of the individual mir-465 family members, we also transfected mir-465a, mir-465b, and mir-465c mimics independently with the GHR reporter plasmid. We found that each mir-465 mimic caused a ~50% reduction in luminescence, which was comparable to the reduction seen when all three miRNA mimics were transfected together (Figure 4d). These results indicate that all three mir-465 family members can target the predicted site in the GHR 3'UTR leading to a reduction in GHR mRNA expression.

2.3 | The effects of miR-465 family expression on GH signaling

A reduction in GHR is expected to result in decreased GH signaling. Activation of the GHR leads to phosphorylation of the tyrosine kinase Janus kinase 2 (JAK2), which subsequently phosphorylates the transcription factor signal transducer and activator of transcription 5 (STAT5). Phosphorylated STAT5 then translocates to the nucleus where it activates the transcription of growth hormone response genes including insulin-like growth factor 1 (IGF-1), insulin-like growth factor-binding protein 3 (IGFBP3), and insulin-like growth factor acid-labile subunit (IGFALS). To investigate the effect of mir-465 family expression on GH signaling, AML12 cells were grown in serum-free medium for 4 days to allow for expression of liver-specific genes (Supporting Information Figure S5), followed by transfection with the mir-465c mimic. GH was added 48 hr posttransfection and activation of the GH signaling pathway was followed for 4 hr. Both control cultures and cultures expressing mir-465c mimic showed an immediate

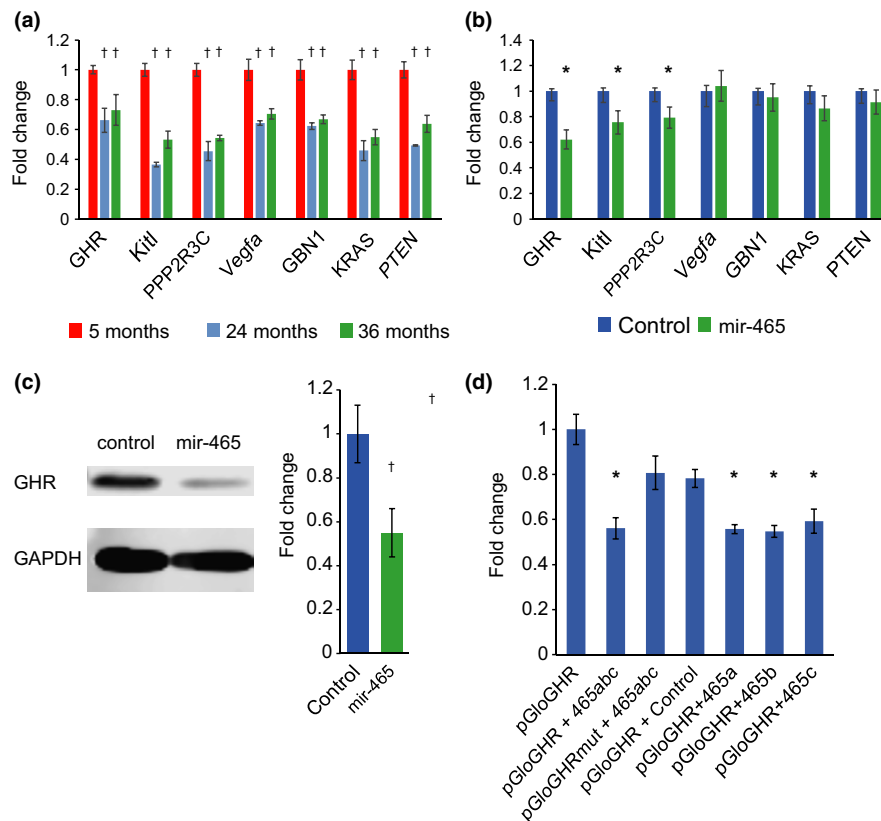


FIGURE 4 Targets of the miRNAs in the X-chromosome cluster show reduced expression. (a) Quantitative PCR analysis of potential targets of the mir-465 family that are reduced with age in RNA-seq analysis verifies that expression of GHR, Kitl, PPP2R3C, Vegfa, GBN1, KRAS, and PTEN is downregulated between 27% and 64% at 24 and 36 months of age in liver. Each bar represents the average of five (24 months) or six (5 and 36 months) animals, run in triplicate. (b) GHR, Kitl, and PPP2R3C mRNA levels are significantly reduced by 38%, 25%, and 21%, respectively, in AML12 cells transfected with either pmir465abc or mir-465c mimics when compared to control cells. mRNA expression levels of the other targets that are reduced with age in liver are not affected by mir-465. Each bar represents the average expression value of five independent experiments. (c) Western blot analysis revealed a 50% reduction in GHR protein in AML12 cells 48 hr following transfection with pmir465abc or the mir-465c mimic. The blot image is from a representative experiment, and the bar graph represents the average normalized intensity values of four independent experiments. (d) A 50% reduction in luminescence signal was observed in HEK-293T cells cotransfected with a reporter plasmid containing the GHR 3'UTR (pGloGHR) along with the mir-465 family (465abc) when compared to cells transfected with pGloGHR only. A significant reduction in luminescence signal was not seen in cells cotransfected with a reporter plasmid containing a mutated target site (pGloGHRmut) and 465abc, or in cells cotransfected with pGloGHR and a negative control miRNA. Individually, members of the mir-465 family caused a reduction in luminescence similar to the reduction seen with the combination of all three family members. Each bar represents the average normalized luminescence value from six independent experiments. All error bars represent standard errors. All p -values were calculated using two-tailed Student's t test. * $p < 0.05$, † $p < 0.01$

response to GH by increasing phosphorylation of JAK2 within 5 min (Figure 5a). However, cells containing mir-465c mimic had a muted response indicated by a twofold increase in JAK2 phosphorylation, as compared to a fivefold increase seen in control cells. A similar reduction in signaling was seen in STAT5 phosphorylation, where control cells showed a much larger increase in STAT5 phosphorylation (43-fold after 15 min) when compared to cells containing the mir-465c mimic (15-fold after 15 min) (Figure 5b). These results show that a reduction in the GHR resulting from the presence of mir-465c leads to an attenuation of GH signaling in response to GH stimulation.

The primary response to GH signaling is an increase in IGF-1 expression. To determine whether the reduction in the GHR and the GH signaling response in the presence of mir-465 leads to reduced IGF-1 expression, we analyzed IGF-1 mRNA expression following GH stimulation. We found an attenuation in IGF-1 mRNA expression in

AML12 cells transfected with either pmir465abc or the mir-465c mimic (Figure 5c). In control cells, IGF-1 expression increases threefold within 1 hr of GH stimulation. This induction was only 1.5-fold in cells transfected with pmir465abc or the mir-465c mimic. IGF-1 circulates in the plasma as a complex with IGF-1-binding protein 3 (IGFBP3) and IGF-1 acid-labile subunit (IGFALS). Expression of both of these IGF-1-associated proteins increases upon GH stimulation. We saw a twofold increase in IGFBP3 expression in control cells within 2 hr of GH stimulation, which continued to increase at 4 hr post-GH stimulation. However, in cells transfected with the mir-465c mimic, there was no increase in expression during this time frame. Contrarily, we did not see a significant increase in IGFALS expression in either control or mir-465c-treated cells. These results indicate that the reduction in GHR in the presence of mir-465 family members leads to a reduction in IGF-1 and IGFBP3 expression in response to GH stimulation.

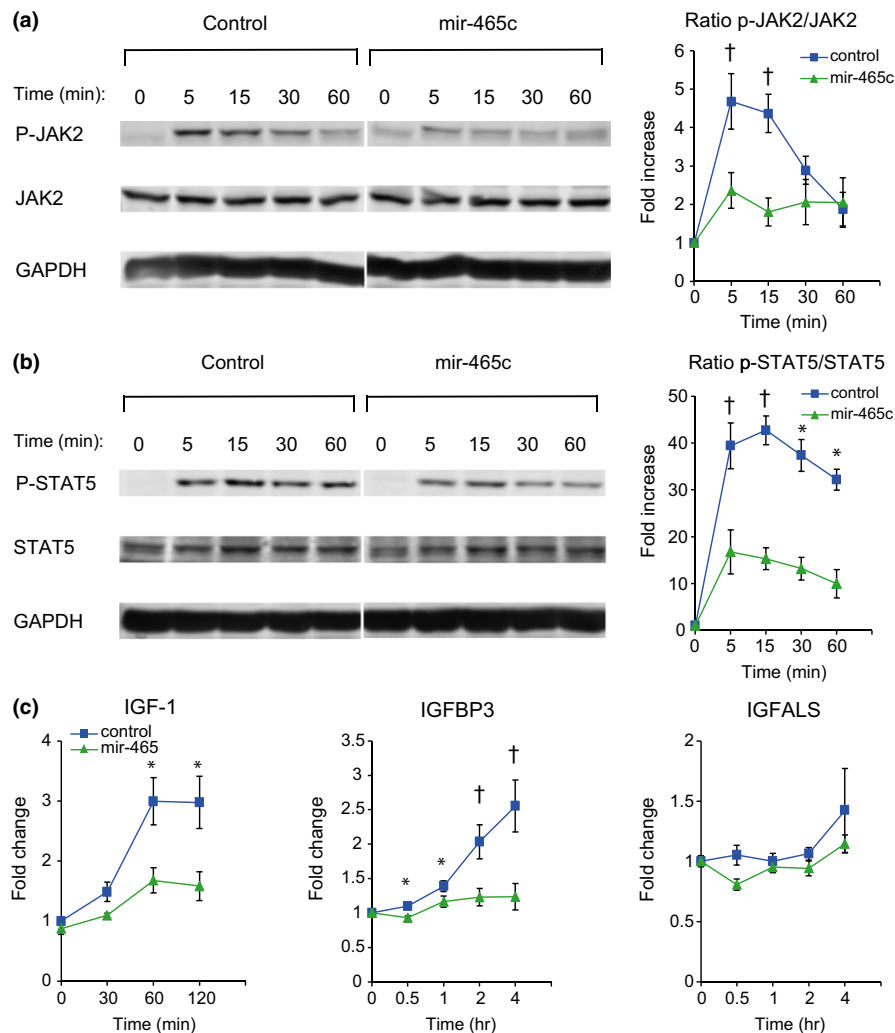


FIGURE 5 Time course of GH signaling in AML12 cells. The effect of reduced GHR expression by mir-465c on GH signaling in the cultured mouse liver cell line AML12. (a) Transfection of AML12 cells with a mir-465c mimic led to reduced JAK2 phosphorylation following GH stimulation. In control cells, the ratio of p-JAK2/Jak2 increased almost fivefold within 5 min of GH addition and stayed elevated for 15 min following GH treatment. JAK2 phosphorylation was attenuated in cells transfected with a mir-465c mimic. The ratio of p-JAK2/JAK2 increased only twofold 5 min. following GH stimulation and remained low over the next 60 min. (b) Phosphorylated active JAK2 phosphorylates STAT5. In control cells, the ratio of p-STAT5/STAT5 increased 40-fold within 5 min. following GH stimulation and remained elevated over the next 60 min. AML12 cells transfected with the mir-465c mimic showed an attenuated response with the ratio of pSTAT5/STAT5 increasing only 15-fold within 5 min. following GH stimulation and remaining low over the next 60 min. (c) GH signaling results in increased IGF-1 expression. IGF-1 expression increased threefold within 60 min of GH stimulation in control cells. Cells transfected with the mir-465c mimic showed an attenuated response, with IGF-1 expression increasing 1.5-fold 60 min. following GH stimulation. Control cells showed a 2-fold increase in IGFBP3 expression within two hours of GH stimulation. No increase in IGFBP3 expression was observed in cells transfected with the mir-465c mimic. IGFBP3 expression did not increase following GH stimulation in control cells or in cells transfected with the mir-465c mimic. Time = time following GH addition. All error bars represent the standard errors. All *p*-values were calculated using two-tailed Student's *t* test. **p* < 0.05, †*p* < 0.01

3 | DISCUSSION

We performed small transcriptome analysis to identify changes in small RNA expression in aged male mouse liver. We found 56 miRNAs that changed expression levels with age. The most prominent change we found was the upregulation of a cluster of 18 miRNA genes present in a 60-kb region of the X-chromosome. This derepression was most pronounced in aged mouse liver, but also occurred in other tissues including skeletal muscle and brain. This cluster of 18 miRNA genes is expressed in the testes and the majority of the miRNAs show minimal expression in other tissues

(Landgraf et al., 2007; Ro et al., 2007). The derepressed miRNAs include members of the mir-465 family: one copy of mir-465a and two copies each of mir-465b and c. These five miRNAs appear to be the result of duplication events as they only vary by 1–3 nucleotides, have the same seed sequence, and are predicted to recognize the same mRNA targets. Here, we show that all members of the mir-465 family target the 3'UTR of the GHR mRNA equally, indicating that all three mir-465 family members have equal specificity for the GHR mRNA 3'UTR. We believe that this is the first report on the functionality of this family of miRNAs. While the data presented here are

specific for male mice, we expect to see similar, if not more prominent, upregulation of the X-chromosomal miRNA cluster in female mice due to loss of repression of sequences on the inactivated X-chromosome with age. Telomere shortening, which is associated with organismal aging, was shown to contribute to a loss of H3K27me3 silencing and an upregulation of sequences on the inactivated X-chromosome in female mice (Schoeftner et al., 2009). Future in vivo studies will investigate gender-specific differences in the upregulation of these miRNAs.

The somatotrophic axis in mammals consists of growth hormone-releasing hormone (GHRH), GH, and IGF-1 that work in concert to regulate cell survival and growth. The principal site of GH action is the liver, where it binds to the GHR initiating a signaling cascade through the JAK/STAT pathway resulting in IGF-1 production. In mammals, levels of circulating GH and IGF-1 begin to decline in early adulthood and continue to decline throughout the lifespan in a process called somatopause (Bartke, 2008). A reduction in circulating IGF-1 in adulthood is a major risk factor for the development of a number of age-associated pathologies and diseases, including diabetes, osteoporosis, sarcopenia, cardiovascular disease, dementia, and Alzheimer's disease (Bartke et al., 2016; Barzilai et al., 2012; Laughlin, Barrett-Connor, Criqui, & Kritz-Silverstein, 2004; Lombardi et al., 2005; Toth et al., 2015; Westwood et al., 2014). GH signaling also plays a significant role in regulating hepatic lipid metabolism. A reduction in GH signaling, through either GH deficiency or reduced GHR activity, leads to an upregulation of the fatty acid transporter Cd36, and several genes involved in lipogenesis (*Fasn*, *Scd1*) resulting in hepatic accumulation of triglycerides and lipid, leading to the development of nonalcoholic fatty liver disease (NAFLD; reviewed in Kaltenecker et al., 2018). The data presented here suggest that the increase in expression of the mir-465 family could contribute to the reduction in circulating IGF-1 and to the onset of NAFLD with age.

The mechanism behind the derepression of the cluster of miRNAs is currently unknown and is a direction of future study. Recent work from our laboratory and others established that a global change in chromatin structure occurs with age, characterized by heterochromatic regions of the genome becoming relatively more open and the derepression of repetitive elements present in these regions (Criscione et al., 2016; Criscione, Zhang, Thompson, Sedivy, & Neretti, 2014; De Cecco, Criscione, Peckham, et al., 2013; De Cecco, Criscione, Peterson, et al., 2013; Kreiling et al., 2011; Swanson, Manning, Zhang, & Lawrence, 2013; Wood et al., 2010). In addition, we found that lamin-associated domains dissociate from the nuclear lamina in senescent human diploid fibroblasts (HDF) indicating a loss of repression (De Cecco, Criscione, Peckham, et al., 2013). We found that the cluster of miRNAs is located in a 60-kb gene-poor region of the X-chromosome that contains high numbers of repetitive elements and is enriched in beta-lamin interactions, indicating that it is part of a heterochromatic lamin-associated domain. Therefore, the age-associated relaxation of heterochromatin may lead to expression of these miRNAs, which could subsequently affect the major signaling pathways that are known to be altered with age.

We began to see an increase in expression of mir-465 family members at 12 months of age, and expression continued to increase until 24 months of age. We found that an increase in mir-465 family expression in a mouse liver cell line by transfection of mir-465 mimics led to a reduction in GHR at both the mRNA and protein levels, suggesting that these miRNAs act by inducing mRNA degradation rather than inhibiting translation. The reduction in GHR resulted in reduced IGF-1 and IGFBP3 expression. Contrarily, we did not see an increase in IGFALS, as would be expected to result from GH stimulation (Dai, Scott, & Baxter, 1994; Ooi, Cohen, Tseng, Rechler, & Boisclair, 1997). However, it has been reported that induction of IGFALS following GH stimulation in cultured cell lines is not detected and it is hypothesized that altered chromatin structure in cultured cell lines may contribute to this phenotype (Dai et al., 1994; Ooi et al., 1997). The reduction of IGF-1 and IGFBP3 expression indicates that dysregulation of mir-465 family expression with age may contribute, at least in part, to the reduction in circulating IGF-1 with age in vivo through its action on GHR expression. Future studies in vivo will help elucidate the effects of mir-465 family expression on the somatotrophic axis and healthspan.

The role of GH/IGF-1 signaling on healthspan and lifespan has been studied in a wide range of organisms from invertebrates to humans (Bartke et al., 2016; Barzilai et al., 2012; Brown-Borg, 2015). Interestingly, there is a high degree of conservation in the GH/IGF-1 signaling pathway across species in both its function and effects on healthspan and lifespan. The mir-465 family target site in the 3' UTR of the GHR mRNA is highly conserved across mammals, indicating that it is likely important for regulation of GHR expression (Supporting Information Figure S4). While the mir-465 family has only been identified in rodents to date, the conservation of the target site in the GHR mRNA 3'UTR across mammals, including humans, indicates that there are likely unidentified miRNAs or other regulatory elements that are involved in regulation of GHR expression at this site.

We found two additional targets that are downregulated in the presence of mir-465 family. Kitl, also known as stem cell factor, is a growth factor that binds and activates the receptor tyrosine kinase c-Kit, initiating PI3K signaling. c-Kit signaling has been shown to promote proliferation, migration, survival, and differentiation in a wide range of cell types including hematopoietic progenitors, melanocytes, germ cells, interstitial cells of Cajal, neural progenitors, vascular smooth muscle cells, and epithelial progenitor cells (reviewed in Lennartsson & Ronnstrand, 2012). In addition, mutations in Kitl or c-Kit have been documented in a large number of cancers (Sanger Institute Catalogue of Somatic Mutations in Cancer, 2018). However, c-Kit signaling has not been investigated in liver in the context of aging. Given its role in cell survival and differentiation, a reduction of Kitl expression with age could lead to dysregulation of hepatocyte function.

We also found PPP2R3C is downregulated by the mir-465 family. PPP2R3C is a regulatory subunit of protein phosphatase 2 (PP2A) and serine/threonine-protein phosphatase 5 (PP5; Kono et al., 2002). These phosphoprotein phosphatases are highly conserved in eukaryotes (Brautigan, 2013). PP2A and PP5 regulate signal

transduction pathways that control most cellular processes including DNA replication, transcription, translation, metabolism, cell cycle progression, cell division, development, and apoptosis (Janssens & Goris, 2001; Kono et al., 2002; Perrotti & Neviani, 2013). In addition, PPP2R3C regulates P-glycoprotein (P-gp), an efflux pump that removes toxic compounds from the cell, including chemotherapy drugs making them less effective (Katayama, Yamaguchi, Noguchi, & Sugimoto, 2014). The role PPP2R3C plays in liver homeostasis and changes that occur with age have not been investigated, but given the wide range of cellular processes it regulates it is expected that a reduction in expression could alter cellular function.

Only one other miRNA in the cluster, mir-470, has been shown to be expressed outside of the testes. It is expressed during differentiation of embryonic stem cells where it targets Nanog and Oct4 expression (Tay, Zhang, Thomson, Lim, & Rigoutsos, 2008). It is also expressed in the central nervous system. In the adult brain, mir-470 expression is tied to regulation of neuronal firing (van Spronsen et al., 2013). Taken together with our finding that mir-470 and several other members of the X-chromosome cluster are upregulated in the brain with age (Supporting Information Figure S2), and that the miRNAs in the cluster are predicted to influence axon guidance, GABAergic synapse, dopaminergic synapse, and glutamatergic synapse pathways (Table 1), the upregulation of these miRNAs may be important in age-associated changes in neuronal function. In addition, mir-470 is expressed in the hippocampus of the Ames dwarf long-lived mouse where its expression leads to a >30% reduction in IGF-1 receptor (Liang et al., 2011). These results are interesting, when combined with the results presented here they suggest that the cluster of miRNAs may work together to regulate the somatotrophic axis. This hypothesis is further strengthened when you consider that bioinformatic analyses predict 14 of the miRNAs have potential targets in 69 genes involved in PI3K-Akt signaling (Table 1). These include the PI3K 3'UTR that is predicted to be recognized by mir-743a and mir-871, the AKT3 3'UTR that is predicted to be targeted by mir-883b, and the IGF-1 3'UTR that is predicted to be targeted by mir-743a, mir-871, and mir-881. Since all 18 of the miRNAs are upregulated as a cluster, it is possible that they work together in concert to regulate various aspects of PI3K-Akt signaling. Therefore, dysregulation of the X-chromosomal cluster of miRNAs with age may have broader reaching consequences by attenuating several segments of this signaling pathway. It is known that PI3K-Akt signaling is altered in many of the diseases associated with aging. Future studies involving additional miRNAs found in the X-chromosome cluster will provide insight into the role that these miRNAs play in both normal aging and the onset of age-associated pathologies.

4 | EXPERIMENTAL PROCEDURES

4.1 | Mouse tissue

Mouse tissues of strain C57BL/6 were collected in-house from male mice obtained from the NIA Aged Rodent Colonies (<https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook>).

4.2 | Cell lines

The human embryonic kidney cell line HEK-293T and the mouse hepatocyte cell line AML12 (both from ATCC, Manassas, VA, USA) were used in this study. For detailed cell culture procedures, see Supporting Information Data S1.

4.3 | High-throughput sequencing

The small RNA fraction was isolated from young (5 months), old (24 months), and very old (36 months) mouse liver and sequenced on the Illumina HiSeq2000. We previously published the RNA-seq datasets (De Cecco, Criscione, Peterson, et al., 2013). See Supporting Information Data S1 for detailed protocols for both datasets.

4.4 | Luminescence assay

The full-length 3'UTR of the GHR gene was cloned into the pmir-GLO reporter plasmid (Promega, Madison, WI, USA) at the 3' end of the firefly luciferase gene (pGloGHR). Site-directed mutagenesis was used to eliminate the potential mir-465 target site in pGloGHR. HEK-293T cells were cotransfected with miRNA mimics corresponding to mmu-mir-465a, mmu-mir-465b, mmu-mir-465c, or cel-mir-39 (Exiqon, Woburn, MA, USA) and either pGloGHR or the empty vector. After 48 hr, Firefly and Renilla luciferase activity was detected using the DualGlo Luciferase Assay System (Promega) according to the manufacturer's protocol. Detailed protocols can be found in the Supporting Information Data S1.

4.5 | miRNA expression plasmid and mimics

The coding genes of the three members of the mir-465 family were cloned into the mammalian expression vector pcDNA3.1 under the control of the CMV promoter to create pmir465abc. Full descriptions of the primers used (Supporting Information Table S1) and the cloning procedure can be found in the Supporting Information Data S1.

4.6 | GH stimulation

AML12 cells were cultured for 96 hr in culture medium without serum to stimulate the expression of liver-specific genes including the GHR (Supporting Information Figure S5). The medium was replaced with serum-free culture medium containing 500 ng/ml mouse growth hormone (GenScript, Piscataway, NJ, USA). See the Supporting Information Data S1 for more details.

4.7 | Quantitative real-time PCR

Quantification of miRNA expression was performed using cDNA made following the procedure outlined in Balcells, Cirera, and Busk (2011). Details on the RNA isolation, cDNA synthesis, primer design, and cycling parameters can be found in the Supporting Information Data S1.

4.8 | Quantitative immunoblotting

Following GH treatment, cells were harvested, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (MilliporeSigma, Burlington, MA, USA). Membranes were probed with the appropriate primary and secondary antibodies (Supporting Information Table S2). Signals were detected using the Li-Cor Odyssey Clx Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed either with the gel analysis component of ImageJ open source software (<https://imagej.nih.gov/ij/>) or with the Li-Cor Odyssey analysis software. A detailed protocol can be found in the Supporting Information Data S1.

4.9 | Antibodies

Primary and secondary antibodies and dilutions used in this study are outlined in Supporting Information Table S2. Secondary antibodies for immunoblotting were labeled with Odyssey IRDye 680CW or 800CW (Li-Cor Biosciences).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

J.A.K. conceived and designed the study, performed experiments, analyzed data, and wrote the manuscript. A.E.E. performed experiments and analyzed data. B.K., I.M.C.S., G.G.-M., and A.C.Z. performed experiments.

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SUPPORTING INFORMATION

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MicroRNA dysregulation influences growth hormone signaling

Amy E. Elias and Jill A. Kreiling

The somatotrophic axis has been implicated in regulating healthspan and lifespan in a wide range of organisms from invertebrates to humans [1]. The somatotrophic axis consists of growth hormone releasing hormone, growth hormone (GH), and insulin-like growth factor 1 (IGF-1) that work together to regulate cell metabolism, survival and growth. In mammals, GH is released into the circulation by the pituitary gland, and its primary site of action is the liver, where it activates the growth hormone receptor (GHR). Once activated, the GHR initiates JAK2/STAT5 signaling resulting in IGF-1 production. Knocking down GH signaling throughout the entire lifespan in model organisms is associated with an extension of healthspan and lifespan. This appears to be true for humans as well, as centenarians have higher frequencies IGF1R gene polymorphisms that result in altered IGF-1 signaling, and individuals with mutations in the growth hormone receptor gene that give rise to Laron dwarfism have decreased rates of age-associated pathologies, including diabetes and cancer [2]. However, reduced GH signaling in the post-juvenile period appears to have the opposite effect on healthspan and lifespan. A reduction in circulating IGF-1 in adulthood is a major risk factor for the development of many age-associated pathologies, including non-alcoholic fatty liver disease, diabetes, osteoporosis, sarcopenia, cardiovascular disease, dementia, and Alzheimer's disease [3]. Levels of circulating GH and IGF-1 peak in early adulthood, then decline throughout the remaining lifespan in a process called somatopause. The mechanisms surrounding the natural reduction in GH and IGF-1 are not clearly understood. Recently, it was shown that the mir-465 family of miRNAs is upregulated with age in mouse liver and can directly influence GH signaling by targeting the GHR mRNA [4]. The mir-465 family consists of five genes located on the X-chromosome: one copy of mir-465a, and two copies each of mir-465b and mir-465c. Expression of these miRNAs led to a reduction in the GHR at both the mRNA and protein level, and a subsequent reduction in activation of the JAK2/STAT5 signaling pathway resulting in reduced IGF-1 and IGF-1 binding protein 3 (IGFBP3) expression. These findings suggest that the upregulation of the mir-465 family may contribute to the decline in IGF-1 with age.

The mechanisms behind this increase in miRNA expression has yet to be elucidated. The mir-465 family

is part of a cluster of 18 miRNAs that are normally tightly repressed in liver. However, all 18 miRNAs in this cluster are upregulated with age [4]. This cluster is located in a highly heterochromatic region of the X-chromosome that is rich in repetitive elements and devoid of other coding sequences, consistent with constitutive heterochromatin. Chromatin structure undergoes a massive reorganization with age, with a closing of euchromatic regions and an opening of heterochromatic regions [5-8]. These changes in chromatin states correlate with changes in expression in these regions. Retrotransposons and other repetitive elements that are located in regions of constitutive heterochromatin increase in expression with age, likely due to the opening of the heterochromatin [6, 7]. Relaxation of heterochromatin can lead to expression of miRNA genes located in these regions. Given that the X-chromosomal cluster is located a highly heterochromatic region, this mechanism may be responsible, at least in part, for the age-associated increase in expression of the 18 miRNAs, including mir-465 family. The miRNAs located in this cluster are predicted target hundreds of potential mRNAs, therefore, their expression may have broad reaching consequences on cellular function.

Currently, very little is known about the targets of this cluster of miRNAs. Only one other miRNA in the cluster, mir-470, has been studied thus far. It is expressed in the hippocampus of the long-lived Ames Dwarf mouse, where it leads to a reduction in the IGF-1 receptor. Bioinformatic analyses predict that three additional miRNAs in the X-chromosomal cluster (mir-743a, mir-871, and mir-881) may target IGF-1 [4], signifying that the age-associated up-regulation of this cluster of miRNAs may contribute to the dysregulation of the somatotrophic axis. GH signaling is highly intertwined with the PI3K-Akt signaling network, which regulates cell metabolism, proliferation, survival and growth. Alterations in PI3K-Akt signaling has been implicated in the onset of many age-associated pathologies, including cancer, diabetes, and neurodegenerative conditions. Bioinformatic analyses predict 14 of the miRNAs in the X-chromosomal cluster have potential targets in 69 genes involved in PI3K-Akt signaling [4], suggesting that the 18 miRNAs may work together to contribute to the dysregulation of PI3K-Akt signaling with age, including GH signaling. Therefore,

the age-associated upregulation of the X-chromosomal cluster of miRNAs may have impacts beyond GH signaling by influencing multiple pathways involved in cell growth and metabolism. Future studies investigating additional targets of these miRNAs will give insight into the molecular mechanisms involved in the development of aging phenotypes and pathologies, and may provide potential therapeutic targets to counteract these impacts.

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L1 drives IFN in senescent cells and promotes age-associated inflammation

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Retrotransposable elements are deleterious at many levels, and the failure of host surveillance systems for these elements can thus have negative consequences. However, the contribution of retrotransposon activity to ageing and age-associated diseases is not known. Here we show that during cellular senescence, L1 (also known as LINE-1) retrotransposable elements become transcriptionally derepressed and activate a type-I interferon (IFN-I) response. The IFN-I response is a phenotype of late senescence and contributes to the maintenance of the senescence-associated secretory phenotype. The IFN-I response is triggered by cytoplasmic L1 cDNA, and is antagonized by inhibitors of the L1 reverse transcriptase. Treatment of aged mice with the nucleoside reverse transcriptase inhibitor lamivudine downregulated IFN-I activation and age-associated inflammation (inflammaging) in several tissues. We propose that the activation of retrotransposons is an important component of sterile inflammation that is a hallmark of ageing, and that L1 reverse transcriptase is a relevant target for the treatment of age-associated disorders.

The activity of retrotransposable elements (RTEs) can promote aberrant transcription, alternative splicing, insertional mutagenesis, DNA damage and genome instability¹. RTE-derived sequences comprise up to two-thirds of the human genome², although the vast majority were active millions of years ago and are no longer intact. The only human RTE capable of autonomous retrotransposition is the long-interspersed element-1 (L1, also known as LINE-1). However, germline activity of L1 is a major source of human structural polymorphisms³. Increasing evidence points to RTE activation in some cancers, in the adult brain, and during ageing^{4–7}. Cellular defences include heterochromatinization of the elements, small RNA pathways that target the transcripts, and antiviral innate immunity mechanisms⁸. Somatic activation of RTEs with age is conserved in yeast and *Drosophila* and reducing RTE activity has beneficial effects⁸.

Activation of L1 and IFN-I in cellular senescence

We show here that L1 transcription is activated exponentially during replicative senescence of human fibroblasts, increasing 4–5-fold by 16 weeks after cessation of proliferation, which we refer to as late senescence (Fig. 1a, Extended Data Fig. 1a–e). Several quantitative reverse transcription PCR (RT–qPCR) primer pairs were designed to detect evolutionarily recent L1 elements (L1HS–L1PA5; Fig. 1b, Extended Data Fig. 1h). Levels of L1 poly(A)⁺ RNA increased 4–5-fold in late replicatively senescent cells in the sense but not antisense direction throughout the entire element (Fig. 1c). Sanger sequencing of long-range RT–PCR amplicons (Fig. 1b) identified 224 elements that were dispersed throughout the genome; one-third (75, 33.5%) of these were L1HS, of which 19 (25.3%, 8.5% of total) were intact (annotated to be free of open-reading frame (ORF)-inactivating mutations; Extended Data Fig. 1f, g). We also performed 5′ rapid amplification of cDNA ends (RACE) with the same primers and found that most L1 transcripts

upregulated in senescent cells initiated within or near the 5′ untranslated region (UTR) (Extended Data Fig. 2).

L1 elements can stimulate an IFN-I response⁹. We found that α -family interferons (*IFNA* gene cluster on chromosome 9, see Methods) *IFNA* and *IFNB1* were induced to high levels in late senescent cells (Fig. 1d, Extended Data Fig. 1i). Cellular senescence proceeds through an early DNA damage-response phase followed by the senescence-associated secretory phenotype (SASP) response¹⁰. We document here a third and even later phase, characterized by the upregulation of L1 and an IFN-I response (Fig. 1e), which has not been previously noted, probably because most studies have focused on earlier times. Whole transcriptome RNA sequencing (RNA-seq) analysis confirmed that the SASP and IFN-I responses are temporally distinct (Extended Data Fig. 3). The late phase of L1 activation and IFN-I induction was also observed in oncogene-induced senescence (OIS) and stress-induced premature senescence (SIPS) (Fig. 1e, Extended Data Fig. 1j, k).

Mechanisms of L1 activation

To explore how surveillance fails during senescence, we examined three factors: TREX1, RB1 and FOXA1. TREX1 is a 3′ exonuclease that degrades foreign invading DNAs and its loss has been associated with the accumulation of cytoplasmic L1 cDNA¹¹. We found that the expression of TREX1 was markedly decreased in senescent cells (Extended Data Fig. 4a). RB1 has been shown to bind to repetitive elements, including L1 elements, and promote their heterochromatinization¹². We found that the expression of RB1 declined strongly in senescent cells (Fig. 2a), whereas that of other RB family members (*RBL1* and *RBL2*) did not change (Extended Data Fig. 4b). RB1 enrichment in the 5′ UTR of L1 elements was evident in proliferating cells, decreased in early senescence and became undetectable at later times

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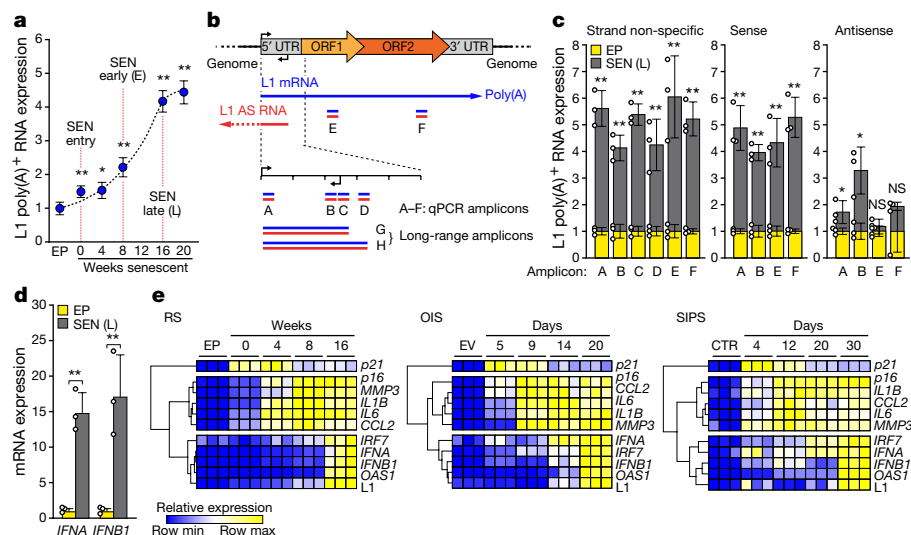


Fig. 1 | Activation of L1, IFN-I and SASP in senescent cells. Gene expression was assessed by RT-qPCR. Poly(A)-purified RNA was used in all L1 assays. **a**, Time course of L1 activation. *P* values were calculated relative to the early passage (EP) control. **b**, Schematic of L1 RT-PCR strategy. Blue, sense; red, antisense (AS). For primer specificity, see Extended Data Fig. 1f–h; for primer design, see Methods. Primers for amplicon F were used in **a** and **e**. **c**, Strand-specific L1 transcription was assessed using amplicons A–F. Transcription from the 5' UTR antisense promoter was also detected. 'SEN (L)' denotes late senescence (16 weeks). **d**, Induction of *IFNA* and *IFNB1* mRNA levels in human fibroblasts. **e**, The temporal induction of genes

associated with DNA damage (*p21* and *p16*, also known as *CDKN1A* and *CDKN2A*, respectively), SASP (*IL1B*, *CCL2*, *IL6* and *MMP3*), and the IFN-I response (*IRF7*, *IFNA*, *IFNB1* and *OAS1*). Row clustering was calculated as 1 – Pearson correlation. OIS, oncogene induced senescence (elicited by Ha-RAS infection); RS, replicative senescence; SIPS, stress-induced premature senescence (gamma irradiation). Controls: EP, early passage; EV, empty vector infected; CTR, non-irradiated. *n* = 3 independent biological samples, repeated in two independent experiments. Data are mean $\pm$ s.d. NS, not significant; **P* $\leq$ 0.05, ***P* $\leq$ 0.01, unpaired two-sided *t*-test. Exact *P* values can be found in the accompanying Source Data.

(Fig. 2a). This coincided with a decrease of histone 3 Lys9 trimethylation (H3K9me3) and H3K27me3 marks in these regions (Extended Data Fig. 4c).

To identify factors that interact with the L1 5' UTR, we examined the ENCODE chromatin-immunoprecipitation followed by sequencing (ChIP-seq) database and found that the pioneering transcription factor FOXA1 binds to this region in several cell lines (Extended Data Fig. 4d). FOXA1 is upregulated in senescent cells¹³ and bound to the central region of the L1 5' UTR (Fig. 2b). Using transcriptional reporters, we found that deletion of the FOXA1-binding site decreased both sense and antisense transcription from the L1 5' UTR¹⁴ (Extended Data Fig. 4e). Hence, the observed misregulation of these three factors in senescent cells could promote the activation of L1 by three additive mechanisms: loss of RB1 by relieving heterochromatin repression; gain of FOXA1 by activating the L1 promoter; and loss of TREX1 by compromising the removal of L1 cDNA.

We thus tested the effects of manipulating RB1, FOXA1 or TREX1 expression in fully senescent cells using lentiviral vectors (Extended Data Fig. 5a, b). Ectopic expression of RB1 suppressed the increased expression of L1, *IFNA* and *IFNB1* in senescent cells, whereas knockdown of RB1 further enhanced their expression (Fig. 2d). RB1 overexpression also restored its occupancy of the L1 5' UTR (Fig. 2c). Conversely, knockdown of FOXA1 reduced its binding to the L1 5' UTR (Extended Data Fig. 4f) and decreased the expression of L1, *IFNA* and *IFNB1*, whereas overexpression of FOXA1 increased the expression of L1, *IFNA* and *IFNB1* (Fig. 2e). Congruent results were also obtained by manipulating TREX1 (Fig. 2g). Hence, each of these factors had a tangible effect on the regulation of L1 and the IFN-I response in senescent cells.

Single or double interventions targeted at these factors elicited only modest changes in L1 and IFN-I expression in growing early passage cells. Although some of these effects were statistically significant, they were overshadowed by a triple intervention (3 $\times$) of RB1 and TREX1 knockdowns combined with FOXA1 overexpression, which resulted in a large induction of L1 and IFN-I expression (Fig. 2f, Extended Data Figs. 4g–i, 5c). Hence, in normal healthy cells all three effectors have to be compromised to unleash L1 effectively.

Consequences of L1 activation

To assess IFN-I activation by L1 in more detail, we examined the expression of 84 genes in this pathway using PCR arrays. We observed a widespread response, with most genes being upregulated (Fig. 2h, Extended Data Fig. 4j, k): 68% (57 out of 84) were significantly upregulated in senescent cells, and 52% (44 out of 84) were upregulated in 3 $\times$ cells. These data verify and further extend the RNA-seq transcriptomic analysis (Extended Data Fig. 3).

Some nucleoside reverse transcriptase inhibitors (NRTIs) developed against HIV have been found to also inhibit L1 reverse transcriptase activity¹⁵. We also developed short hairpin RNAs (shRNAs) against L1, two of which reduced transcript levels by 40–50% and 70–90% in deeply senescent and 3 $\times$ cells, respectively (Extended Data Fig. 5g). Amounts of ORF1 protein were correspondingly reduced in deeply senescent cells (Extended Data Fig. 5h). Finally, the shRNAs also reduced the retrotransposition of recombinant L1 reporter constructs (Extended Data Fig. 5k).

Cells devoid of TREX1 display cytoplasmic L1 DNA, the accumulation of which can be inhibited with NRTIs¹¹. Although the lack of BrdU incorporation is a canonical feature of senescent cells (Extended Data Fig. 1b), longer term labelling revealed DNA species that were predominantly cytoplasmic and highly enriched for L1 sequences (Extended Data Fig. 6a, b). The synthesis of cytoplasmic L1 DNA could be almost completely blocked by the NRTI lamivudine (also known as 3TC) or by shRNA to L1 (Fig. 3a, c). An antibody to DNA–RNA hybrids detected a cytoplasmic signal in senescent cells that largely colocalized with ORF1 protein and turned into a single-stranded DNA (ssDNA) signal after RNase digestion (Extended Data Fig. 6c). Analysis of BrdU-labelled L1 sequences in senescent cells showed them to be localized throughout the L1 element (Extended Data Fig. 6d, e). A relative increase of L1HS sequences in total cellular DNA can also be detected by a qPCR assay^{6,16}. 3TC in the range of 7.5 to 10 μ M completely blocked this increase in senescent cells and also quenched the activity of a L1 retrotransposition reporter (Extended Data Fig. 5d, e).

L1 knockdown with shRNA or treatment of cells with 3TC significantly reduced interferon levels, as well as reducing the IFN-I response more broadly in both late senescent and 3 $\times$ cells (Fig. 3b, Extended

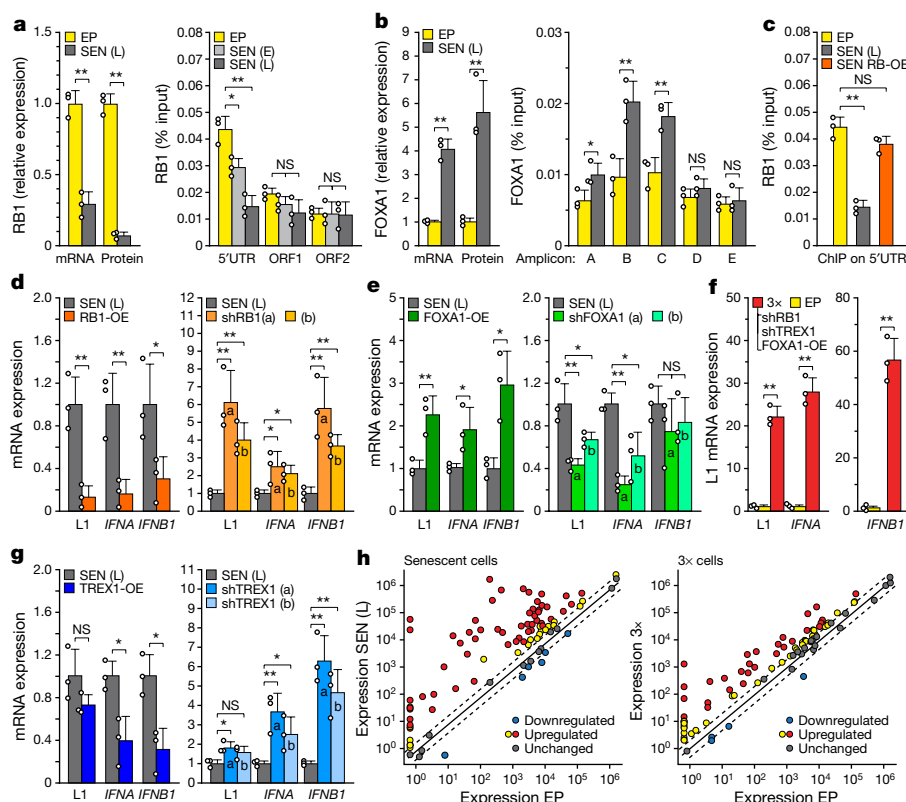


Fig. 2 | Regulation of L1 activation and IFN-I induction. **a, b,** Expression and ChIP of RB1 (**a**) and FOXA1 (**b**). Left, expression was measured by RT-qPCR and immunoblotting. Right, binding to L1 elements was assessed with ChIP-qPCR. For primer locations see Fig. 1b. RB1: 5' UTR, ORF1 and ORF2, primers for amplicons A, E and F, respectively. FOXA1: primers for amplicons A–E. qPCR was normalized to input chromatin. 'SEN (E)' denotes early senescence (8 weeks). For gel source data, see Supplementary Fig. 1. **c,** RB1 was overexpressed (OE), and its binding to 5' UTR of L1 elements was assessed by ChIP-qPCR (amplicon A). **d, e, g,** RB1 (**d**), FOXA1 (**e**) or TREX1 (**g**) were overexpressed or ablated with shRNAs (shRB1, shFOX1 or shTREX1), and the effects on mRNA expression of L1, *IFNA* and *IFNB1* were determined by RT-qPCR of poly(A)-purified RNA. In all cases, lentiviral vectors were used to deliver the interventions directly into senescent cells at 12 weeks (Extended Data Fig. 1a, point D), and cells were collected for analysis 4 weeks later (point E,

16 weeks). Controls were uninfected senescent cells obtained at the same time (point E, 16 weeks). Two distinct shRNAs (**a** and **b**) were used for each gene. Primers for amplicon F were used for L1. **f,** Activation of L1, *IFNA* and *IFNB1* mRNA expression after the triple intervention (3×) using shRB1 (**a**), shTREX1 (**a**) and FOXA1-OE in early passage cells. Lentiviral infections were performed sequentially with drug selections at each step (shRB1, puromycin; shTREX1, hygromycin; FOXA1-OE, blasticidin). **h,** Expression of IFN-I pathway genes was determined with the RT² Profiler PCR array (Qiagen). Normalized mean expression is shown for all 84 genes in the array. Red symbols denote significantly upregulated genes. Dashed lines demarcate the $\pm$ twofold range. $n = 3$ independent biological samples, repeated in two independent experiments (**a, b, h**); $n = 3$ independent experiments (**c–g**). Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided t -test. Exact P values can be found in the accompanying Source Data.

Data Fig. 7a). 3TC in the range of 7.5 to 10 μ M optimally inhibited the IFN-I response, and was the most effective of four NRTIs tested (Extended Data Fig. 5f, j). The relative efficacies of the NRTIs are consistent with their ability to inhibit human L1 reverse transcriptase¹⁵. 3TC also antagonized the IFN-I response in other forms of senescence, OIS and SIPS (Fig. 3e).

We passaged cells in the continuous presence of 3TC from the proliferative phase into deep senescence. 3TC did not significantly affect the timing of entry into senescence, induction of p21 or p16, or the early SASP response (such as upregulation of IL-1 β (Fig. 3f, Extended Data Fig. 7b). However, the magnitude of the later SASP response (such as the induction of CCL2, IL-6 and MMP3) was significantly dampened. Treatment with L1 shRNA also reduced the expression levels of IL-6 and MMP3 in late senescent cells (Extended Data Fig. 6f). Hence, although L1 activation and the ensuing IFN-I response are relatively late in onset, they contribute importantly to the mature SASP and pro-inflammatory phenotype of senescent cells.

3TC did not affect L1 transcript levels (Extended Data Fig. 5i), suggesting that the IFN-I response is triggered by L1 cDNA. As predicted by this model, knockdown of the cytosolic DNA-sensing pathway components cGAS or STING¹⁷ inhibited the IFN-I response in both late senescent and 3× cells (Extended Data Figs. 5l, 7c, d), and also downregulated the SASP response in late senescent cells (Extended Data Fig. 7e).

NRTIs alkyl-modified at the 5' ribose position cannot be phosphorylated and hence do not inhibit reverse transcriptase enzymes; however, they possess intrinsic anti-inflammatory activity by inhibiting P2X7-mediated events that activate the NLRP3 inflammasome pathway¹⁸. Tri-methoxy-3TC (also known as Kamuvudine-9 and K-9), at 10 μ M or 100 μ M, did not inhibit the IFN-I response in either late senescent or 3× cells (Extended Data Fig. 7f). Hence, the effect of 3TC on the IFN-I pathway requires reverse transcriptase inhibition. At high concentrations (100 μ M), K-9 had some inhibitory activity on markers of inflammation, as reported previously¹⁸ (Extended Data Fig. 7g).

To test the role of interferon signalling in SASP, we inactivated the IFN α and IFN β receptor genes (*IFNAR1* and *IFNAR2*) using CRISPR-Cas9. Effective ablation of IFN-I signalling was achieved in both early passage and deep senescent cells (Extended Data Fig. 5m). In both replicative and SIPS forms of senescence, loss of interferon signalling antagonized late (CCL2, IL-6 and MMP3) but not early (IL-1 β) SASP markers (Fig. 3d). This further demonstrates that IFN-I signalling contributes to the establishment of a full and mature SASP response in senescent cells.

Activation of L1 in human and mouse tissues

Activation of L1 expression in human cancers has been detected with an ORF1 antibody⁴. The same reagent showed widespread ORF1

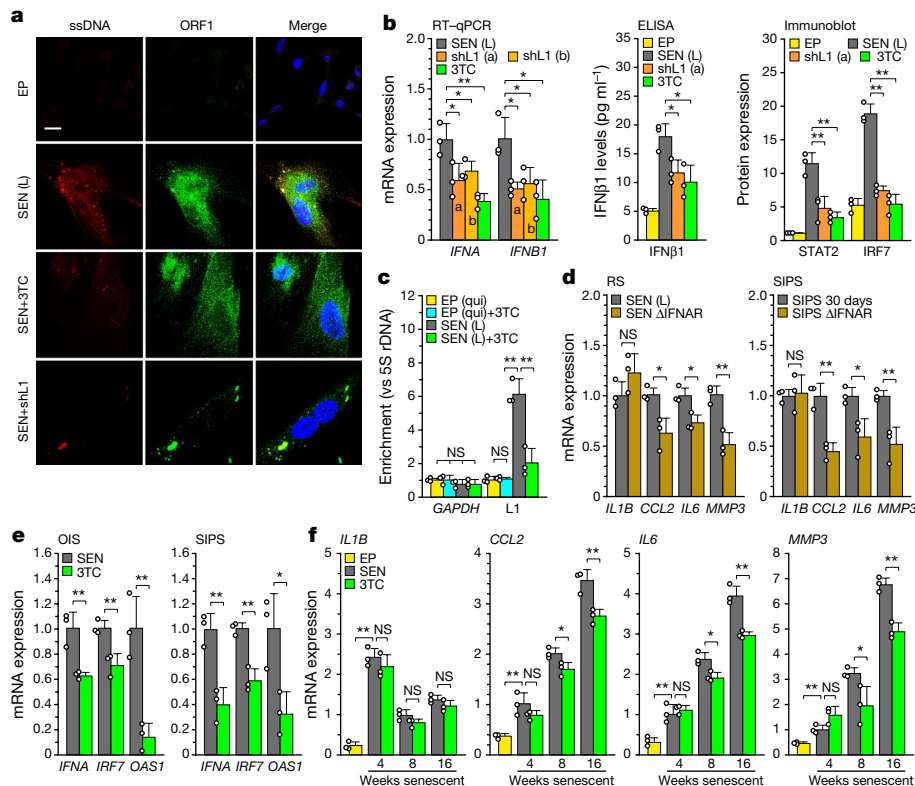


Fig. 3 | Ablation of L1 relieves IFN-I activation and blunts the SASP response. **a**, Cells were examined by immunofluorescence microscopy using antibodies against ssDNA or L1 ORF1 protein. Note the bright cytoplasmic ssDNA puncta in senescent cells that colocalized with prominent puncta of ORF1. The experiment was independently repeated three times with similar results. Scale bar, 10 μ m. **b**, Senescent cells were treated with L1 shRNAs (using lentiviral vectors as described in Fig. 2d, e, g) or with 3TC (7.5 μ M) between 12 and 16 weeks of senescence. Effects on the IFN-I response were determined by RT-qPCR, ELISA or immunoblotting. For gel source data, see Supplementary Fig. 1. **c**, Cells were labelled with BrdU for 2 weeks (with or without 7.5 μ M 3TC), labelled DNA was immunoprecipitated, and its L1 sequence content was quantified using a TaqMan multiplex qPCR assay¹⁶ (Fig. 1b, amplicon F). ‘EP (qui)’ denotes early passage quiescent cells. **d**, Left, replicatively senescent cells. *IFNAR1* and *IFNAR2* genes were mutagenized using the CRISPR–Cas9 system delivered with lentivirus vectors directly into

senescent cells. As with shRNA interventions, cells were infected at 12 weeks and collected at 16 weeks of senescence (Fig. 2d, e, g, Methods). Right, SIPS cells. CRISPR–Cas9 intervention was performed in early passage cells and a validated clone was irradiated to induce SIPS. **e**, OIS (left) and SIPS (right) were induced as in Fig. 1d and cells were collected 20 (OIS) or 30 (SIPS) days later. 3TC (7.5 μ M) was present throughout. IFN-I gene expression (*IFNA*, *IRF7* and *OAS1*) was measured by RT-qPCR. **f**, Cells were serially passaged into replicative senescence with 3TC (10 μ M) present throughout, and the temporal induction of SASP response genes (*IL1B*, *CCL2*, *IL6* and *MMP3*) was assessed. $n = 3$ independent experiments (**b–d**, **f**); $n = 3$ independent biological samples, repeated in two independent experiments (**e**). Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided t -test (**b**, **d–f**) or one-way ANOVA with Tukey’s multiple comparisons test (**c**). Exact P values can be found in the accompanying Source Data.

expression in both senescent and $3\times$ cells (Extended Data Fig. 8a, c, f). In skin biopsies of aged human individuals, we found that 10.7% of dermal fibroblasts were positive for the senescence marker p16, which is in the range documented in ageing primates¹⁹ (Extended Data Fig. 8b, d, f, h). Some of the p16-positive dermal fibroblasts were also positive for ORF1 (10.3%). Notably, we did not observe ORF1 in the absence of p16 expression. We also detected, at the single-cell level, the presence of phosphorylated STAT1, consistent with the presence of interferon signalling in the tissue microenvironment²⁰ (Extended Data Fig. 8b, e, g). Hence, a fraction of senescent cells in normal human individuals displays activation of L1, consistent with these events accumulating during the progression of senescence.

We next examined mice and found that L1 mRNA was progressively upregulated with age in several tissues (Extended Data Fig. 10g). The detected L1 RNA sequences were predominantly sense strand, represented throughout the element, and all three active L1 families were detectable (Extended Data Fig. 6g, h). At the protein level, the frequency of L1 ORF1-positive cells increased in tissues with age (Fig. 4a). Regions of ORF1 staining colocalized with the activity of senescence-associated β -galactosidase (SA- β -Gal) (Fig. 4b). Notably, we found that several IFN-I response genes (*Ifna*, *Ir7* and *Oas1*) as well as pro-inflammatory and SASP markers (*Il6*, *Mmp3* and *Pai1* (also known as *Serpine1*)) were upregulated in the tissues of old (26 months)

mice (Fig. 4c, Extended Data Fig. 9). We also observed an increase in L1 expression and IFN-I response genes (*Ifna* and *Oas1*) in an experimentally induced model of cellular senescence (young mice subjected to sublethal irradiation; Fig. 4d).

We treated old mice (26 months) for two weeks with 3TC (administered in water at human therapeutic doses) and found a broad and significant downregulation of the IFN-I response and alleviation of the SASP pro-inflammatory state (Fig. 4c; see Extended Data Fig. 9 and Supplementary Table 7 for the full dataset). The expression of L1 and *p16* (also known as *Cdkn2a*) mRNA was weakly downregulated, but in most cases did not reach statistical significance. K-9 did not affect either the IFN-I or the SASP responses. Immunofluorescence analysis of tissue sections confirmed that senescent cells expressed SASP markers, and ORF1-expressing cells activated IFN-I signalling (Extended Data Fig. 10a–c). Treatment with 3TC significantly reduced both the IFN-I and SASP responses, but not L1 expression or the presence of senescent cells. Hence, NRTIs can be categorized as ‘senostatic’ agents, in contrast to ‘senolytic’ treatments that remove senescent cells from tissues^{21,22}.

Decreased adipogenesis²³ and thermogenesis²⁴ are features of natural ageing and both were increased in old mice by 2 weeks of 3TC treatment (Extended Data Fig. 10d–f). Longer term treatments (from 20 to 26 months of age) were effective at opposing several known phenotypes

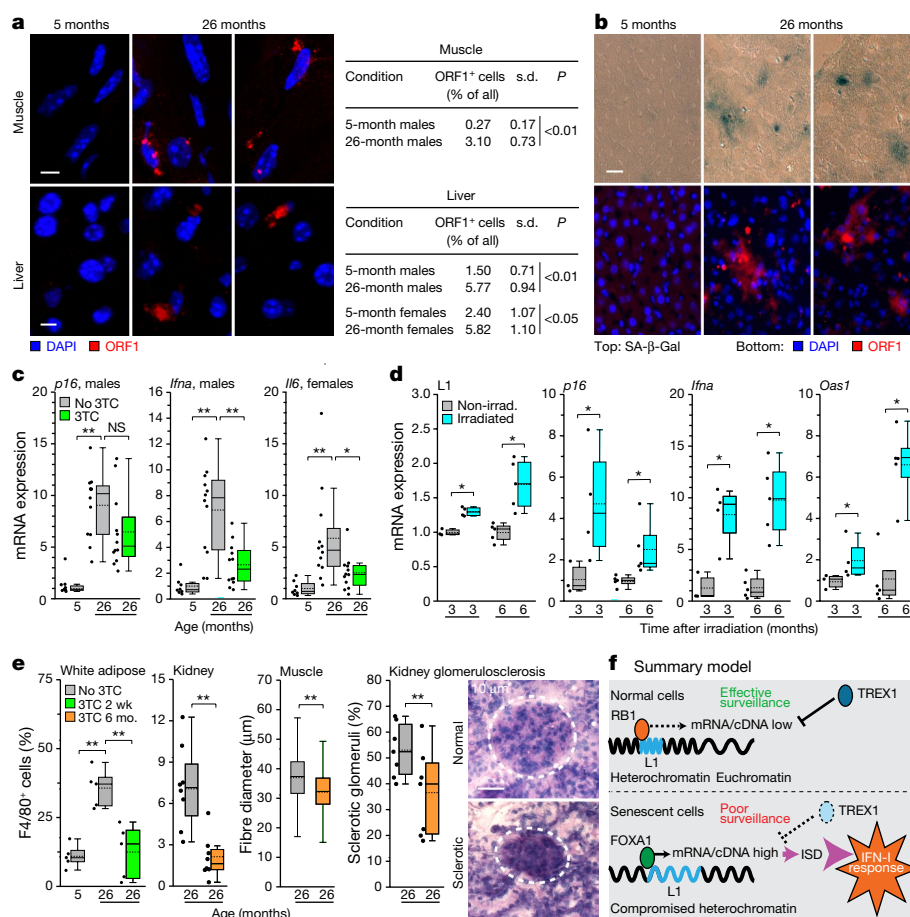


Fig. 4 | L1 elements are activated with age in mouse tissues and the IFN-I pro-inflammatory response is relieved by NRTI treatment. **a**, Presence of L1 ORF1 protein in tissues was examined by immunofluorescence microscopy. Quantification of ORF1-expressing cells is shown in the right panel; 3 mice and at least 200 cells per mouse were scored for each condition. Scale bar, 4 μ m. **b**, Activation of L1 in senescent cells was examined by co-staining for SA- β -Gal activity and ORF1 protein by immunofluorescence (male liver, 5 and 26 months). Scale bar, 4 μ m. The experiment was repeated three times independently with similar results. **c**, Mice were administered 3TC (2 mg ml⁻¹) in drinking water at the indicated ages for two weeks and euthanized after treatment. Expression of *p16*, an IFN-I response gene (*Ifna*), and a marker of a pro-inflammatory state (*Il6*) were assessed in aged adipose tissue by RT-qPCR. See Extended Data Fig. 9 for additional tissues and genes. The box plots in **c** and all subsequent panels show the range of the data (whiskers), 25th and 75th percentiles (box), means (dashed line), and medians (solid line). Each point represents one mouse. *n* = 8 mice at 5 months; *n* = 12 mice at 26 months; *n* = 6 mice at 29 months. **d**, Six-month-old mice were non-lethally irradiated and the expression of L1, *p16* and representative IFN-I

response genes (*Ifna*, *Oas1*) was assessed by RT-qPCR at the indicated times after irradiation in adipose tissue of male mice. Non-irradiated, *n* = 3 mice at 3 months, *n* = 5 mice at 6 months; irradiated, *n* = 4 mice at 3 months, *n* = 5 mice at 6 months. **e**, Macrophage infiltration into white adipose tissue and kidney was scored as F4/80-positive cells (percentage of total nuclei). *n* = 5 mice (adipose); *n* = 8 mice (kidney). Skeletal muscle fibre diameter was measured (Methods) and plotted as an aggregate box plot. *n* = 5 mice per group, 500 fibres total. Glomerulosclerosis was scored in periodic acid-Schiff (PAS)-stained sections (Methods) as the sum of all glomeruli with a score of 3 or 4 divided by the total. *n* = 7 mice per group, 40 glomeruli per animal. 3TC treatment was 2 weeks for white adipose and 6 months (20–26 months) for other tissues. Dashed circle demarcates a single glomerulus. Scale bar, 50 μ m. **f**, Breakdown of L1 surveillance mechanisms leads to chronic activation of the IFN-I response. ISD, interferon-stimulatory DNA pathway. **P* ≤ 0.05, ***P* ≤ 0.01, unpaired two-sided *t*-test (**a**, **d**, **e**), or one-way ANOVA with Tukey's multiple comparisons test (**c**, **e** white adipose). Exact *P* values can be found in the accompanying Source Data.

of ageing: macrophage infiltration of tissues (a hallmark of chronic inflammation^{23,25}), glomerulosclerosis of the kidney²⁶, and skeletal muscle atrophy²⁷ (Fig. 4e). Macrophage infiltration of white adipose tissue was especially responsive, returning to youthful (5-month) levels with only 2 weeks of 3TC treatment.

The activation of endogenous L1 elements and the ensuing robust activation of an IFN-I response is a phenotype of senescent cells, including naturally occurring senescent cells in tissues. This phenotype evolves progressively during the senescence response and seems to be an important, but until now unappreciated, component of SASP. We show that the expression of three regulators, RB1, FOXA1 and TREX1, changes during senescence, and that these changes are both sufficient and necessary to allow the transcriptional activation of L1 elements (Fig. 4f). Hence, several surveillance mechanisms need to be defeated to unleash L1, which underscores the importance of keeping these

elements repressed in somatic cells. We anticipate that future work will uncover additional mechanisms and failure points that can lead to the activation of endogenous RTEs.

The activation of innate immune signalling in response to L1 activation during cellular senescence and ageing proceeds through the interferon-stimulatory DNA pathway. Cytoplasmic DNA can originate from several sources, such as mitochondrial DNA released from stressed mitochondria²⁸ or cytoplasmic chromatin fragments released from damaged nuclei^{29,30}. Our results suggest that L1 cDNA is an important inducer of IFN-I in senescent cells. Notably, NRTI treatment effectively antagonized not only the IFN-I response but also more broadly reduced age-associated chronic inflammation in multiple tissues.

Sterile inflammation, also known as 'inflammaging', is a hallmark of ageing and a contributing factor to many age-related diseases^{31,32}.

We propose that the activation of L1 elements (and possibly other RTEs in the mouse) promotes age-associated inflammation, and that the L1 reverse transcriptase is a relevant target for the development of drugs to treat age-associated disorders.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-018-0784-9>

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Author contributions J.M.S. conceived the study. J.M.S. and M.D. designed the experiments. M.D. and T.I. maintained cell cultures and performed lentiviral interventions. M.D. performed RT-qPCR in cell culture and mouse tissues with help from M.S. and G.B. T.I. and A.C. performed immunoblots. M.D. performed all immunofluorescence analyses. N.J.S., S.W.C. and A.E.E. did the bioinformatics and statistics. A.P.P. performed immunohistochemistry and tissue histology. E.M.A. generated and validated antibodies against mouse ORF1. O.L. irradiated mice and collected tissues. J.A. and K.A. provided NRTI analogues. P.E.S. provided human samples. J.D.B., C.B., J.A., K.A., A.S., V.G., P.E.S., S.L.H., N.N. and J.M.S. contributed to personnel supervision, data interpretation and critical analysis. J.M.S. and M.D. wrote the manuscript with feedback from all authors.

Competing interests J.A. is a cofounder of iVeena Holdings, iVeena Delivery Systems, and Inflammasome Therapeutics, and has been a consultant for Allergan, Biogen, Boehringer-Ingelheim, Janssen, Olix Pharmaceuticals, and Saksin LifeSciences in a capacity unrelated to this work. J.A. and K.A. are named as inventors on patent applications on macular degeneration filed by the University of Kentucky or the University of Virginia. J.D.B. is a founder and Director of Neochromosome, Inc., the Center of Excellence for Engineering Biology, and CDI Labs, Inc. He serves on the Scientific Advisory Boards of Modern Meadow, Inc., Recombinetics, Inc., and Sample6, Inc.

Additional information

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METHODS

Cell culture. Several different strains of normal human fibroblasts were used in this study. LF1 cells were derived from embryonic lung tissue as described³³. These cells have been in continuous use in our laboratory since their isolation in 1996. Original samples frozen in 1996 and in continuous storage in our laboratory were recovered and used. IMR-90 and WI-38 cells were obtained from the ATCC. None of these cell lines is listed in the International Cell Line Authentication Committee (ICLAC) database. These normal fibroblast cell lines were cultured using physiological oxygen conditions (92.5% N₂, 5% CO₂, 2.5% O₂), in Ham's F-10 nutrient mixture (Thermo Scientific) with 15% fetal bovine serum (FBS; Hyclone). Medium was additionally supplemented with L-glutamine (2 mM), penicillin and streptomycin³⁴. Cell cultures were periodically tested for mycoplasma contamination with MycoAlert Mycoplasma Detection Kit (Lonza).

To obtain replicatively senescent cells, LF1 cultures were serially propagated until proliferation ceased. At each passage, after reaching 80% confluence, cells were trypsinized and diluted 1:4. Hence each passage is equivalent to approximately two population doublings. In early passage cultures, the time between passages was constant at approximately 3 days. As cultures approached senescence, the time between passages gradually increased. An interval of 2–3 weeks indicated that the culture was in its penultimate passage. At this point, after reaching 80% confluence, the cells were replated at a 1:2 dilution, and this time was designated as the last passage (point A in Extended Data Fig. 1a). Some cell growth typically does occur in the next 2–3 weeks, but the cultures do not reach 80%. Under this experimental regimen, most of the cells in the culture enter senescence within a 3–4-week window centred roughly around the time of last passage (grey bar in Extended Data Fig. 1a). At point B (4 weeks), the cultures were trypsinized and replated as described³⁵ to eliminate a small fraction of persisting contact-inhibited cells. Cultures were again replated at point C (8 weeks).

OIS was elicited by infecting proliferating LF1 cells with pLenti CMV RasV12 Neo (gift from E. Campeau, Addgene plasmid 22259). Generation of lentiviral particles and the infection procedure are described below. At the end of the infection, cells were reseeded at 15–20% confluency and selected with G418 (250 µg ml⁻¹) maintained continuously until the end of the experiment. Medium was changed every 3 days until the cultures were harvested at the indicated time points. SIPS was elicited by X-ray irradiation with 20 Gy given at a rate of 87 cGy min⁻¹ in one fraction using a caesium-137 gamma source (Nordion Gammacell 40). Cells were 15–20% confluent at the time of irradiation. Medium was changed immediately after irradiation, and at 3-day intervals thereafter. 293T cells (Clontech) were used to package lentivirus vectors and were cultured at 37 °C in DMEM with 10% FBS under normoxic conditions (air supplemented with 5% CO₂).

Nucleoside reverse transcriptase inhibitors. All NRTIs (lamivudine (3TC); zidovudine (also known as azidothymidine, AZT); abacavir (ABC); emtricitabine (FTC)) used in this study were USP grade and obtained from Aurobindo Pharma. For Trizivir (TZV), its constituents (ABC, AZT and 3TC) were combined in the appropriate amounts. Kamuvudine-9 (K-9)³⁶ was provided by Inflammasome Therapeutics.

Mouse husbandry. Compliance with relevant ethical regulations and all animal procedures was reviewed and approved by the Brown University Institutional Animal Care and Use Committee. C57BL/6J mice of both sexes were obtained from the NIA Aged Rodent Colonies (<https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook>) at 5 and 18 months of age. The 5-month-old mice were euthanized after a short (1-week) acclimatization period, a variety of tissues were collected, snap frozen in liquid nitrogen and stored at -80 °C. The 18-month-old mice were housed until they reached a desired age. Mice were housed in a specific pathogen-free AAALAC-certified barrier facility. All procedures were approved by the Brown University IACUC committee. Cages, bedding (Sani-chip hardwood bedding) and food (Purina Laboratory Chow 5010) were sterilized by autoclaving. Food and water (also sterilized) were provided ad libitum. A light–dark cycle of 12 h was used (7:00 on, 19:00 off). Temperature was maintained at 21 °C, and humidity at 50%. All mice were observed daily and weighed once per week. In a pilot experiment, three cohorts of ten mice each were treated with 3TC dissolved in drinking water (1.5 mg ml⁻¹, 2.0 mg ml⁻¹, 2.5 mg ml⁻¹) continuously from 18 months until euthanization at 24 months. The fourth (control) cohort was provided with the same water without drug. No significant differences in behaviour, weight or survival were observed between the four cohorts during the entire experiment. Once during the experiment (at 20 months of age) the mice were subjected to a single tail bleed of approximately 70 µl. The collected plasma was shipped to the University of North Carolina CFAR Clinical Pharmacology and Analytical Chemistry Core for analysis of 3TC. For the 2 mg ml⁻¹ cohort, the concentration of 3TC in plasma averaged 7.2 µM. This dose of drug was chosen for further experiments to mimic the human HIV therapeutic dose (300 mg per day, 5–8 µM in plasma)³⁷. For all experiments, mice were aged in house until they reached 26 months of age. They were then assigned randomly to two cohorts by a technician that was blinded to the appearance or other characteristics of the mice. One cohort

was treated for 2 weeks with 2 mg ml⁻¹ of 3TC in drinking water, and the other (control) cohort with same water without drug, administered in the same manner. At the end of the treatment period, all mice were euthanized and collected for tissues as described above. All mice in both cohorts were included in all subsequent analyses. The experiment was performed on separate occasions with male and female mice. Non-lethal total body irradiation (6 Gy) was performed as described previously³⁸ and tissue specimens were shipped to Brown University on dry ice.

PCR. The ABI ViiA 7 instrument (Applied Biosystems) was used for all experiments. qPCR of DNA was performed using the Taqman system (Applied Biosystems) as described previously¹⁶. Purified genomic DNA (100 pg) was used with the indicated primers (Supplementary Table 1). RT-qPCR of RNA was performed using the SYBR Green system (Applied Biosystems). Polyadenylated RNA was used in all experiments assessing transcription of L1 elements, and total RNA was used for all other genes. Total RNA was collected using the Trizol reagent (Invitrogen). Poly(A) RNA was isolated from total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Total RNA (1 µg) or poly(A) RNA (10 ng) was reverse-transcribed into cDNA in 50-µl reactions using the Taqman kit (Applied Biosystems). To assess strand-specific transcription, the random primers in the reverse transcription reaction were substituted with a strand-specific primer to the target RNA. One microlitre of each reverse transcription reaction was used in subsequent qPCR reactions. *GAPDH* was used as the normalization control in experiments with human cells. The arithmetic mean of *Gapdh* and two additional controls (*Hsp90* (also known as *Hsp90ab1*) and *Gusb*) was used for normalization of RT-qPCR experiments with mouse tissues, with the exception of liver that was normalized to *Hsp90* and *Gusb*. For measuring L1 transcription, poly(A) RNA samples were exhaustively digested with RNase-free DNase (Qiagen) before the synthesis of cDNA⁶. Effectiveness of the DNase digestion was assessed using controls that omitted the RT enzyme.

Design of PCR primers. Primer sets 1 to 5 (Supplementary Table 1, amplicons A to E in Fig. 1b) to human L1 were designed to preferentially amplify elements of the human-specific L1HS and evolutionarily recent primate-specific L1PA(2–6) subfamilies, as follows. First, the consensus sequences of L1HS and L1PA2 through L1PA6 elements were obtained from Repbase (Genetic Information Research Institute, <http://www.girinst.org/repbase/update/browse.php>). Second, a consensus sequence of these six sequences was generated with the Clustal Omega multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)³⁹. Primer design was then done on the overall consensus with Primer3 and BLAST using the NCBI Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/primer-blast/>)⁴⁰. L1 primer pairs were evaluated for their targets using the In-Silico PCR (<https://genome.ucsc.edu/cgi-bin/hgPcr>) tool against the latest genome assembly (hg38) with a minimum perfect match on 3' end of each primer equal to 15. Primers to *ORF2* (primer set 6, amplicon F in Fig. 1b) were developed previously¹⁶ to preferentially target L1HS. Primers to assess transcription of active mouse L1 elements (primer set 37, Supplementary Table 1) were designed on the combined consensus sequence of the L1Mda and L1Tf families obtained from Repbase and validated as described above. L1 primer pairs spanning the full length of these elements (primer sets 48–50) were designed using the same strategy. Primer pairs specific to the three active families of mouse L1 elements (primer sets 51–53) were designed exploiting polymorphisms in the 5' UTR region. RT-qPCR analysis of L1 transcription was performed on poly(A)-purified RNA using the SYBR Green method. For all other (non-L1) genes, whenever possible, primers were separated by at least one intron in the genomic DNA sequence (as indicated in Supplementary Table 1). Primers to the human IFNA family were designed against a consensus sequence of all human IFNA gene sequences (*IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA10*, *IFNA13*, *IFNA14*, *IFNA16*, *IFNA17* and *IFNA21*) generated with the Clustal Omega multiple sequence alignment tool. All primers against mouse targets were designed as described above and are listed in Supplementary Table 1. Sequences of primers corresponding to a consensus of all mouse IFNA family genes, as well as to the *IFNB1* gene, were as described previously⁴¹. To quantify relative L1 genomic copy number (human cells) we used the Taqman multiplex method developed previously¹⁶. These primers are listed as set #6 and set #7 (with their corresponding VIC and 6FAM probes) in Supplementary Table 1.

Chromatin immunoprecipitation. All ChIP experiments were performed using the Chromatrap spin column ChIP kit (Porvair). In brief, 2 × 10⁶ cells were crosslinked in their culture dishes with 1% formaldehyde (10 min, room temperature), quenched with glycine, washed twice with ice-cold PBS (containing protease inhibitors), and finally scraped into a microfuge tube. Cell pellets were resuspended in 0.4 ml of hypotonic buffer and incubated for 10 min on ice. Nuclei were spun down, resuspended in 0.3 ml lysis buffer, and sonicated using a Bioruptor UCD-200 instrument (Diagenode) set to pulse on high (30 s, followed by 30 s rest) for a total time of 10 min. The extracts were centrifuged in a microfuge (top speed, 5 min, 4 °C) to remove debris, the supernatants were transferred to new tubes, and stored at -80 °C. An amount of extract containing 2 µg of DNA was combined with 4 µg of antibody and loaded on a Chromatrap solid phase Protein A matrix.

Immunocomplexes were allowed to form overnight at 4 °C with mild agitation, following which the samples were washed and eluted according to the manufacturer's protocol. Rabbit IgG and 1% input were used as controls. One microlitre of immunoprecipitated DNA was used in each qPCR reaction.

BrdU pull-down. To obtain quiescent cells, proliferating cells were grown to 50% confluence, serum supplementation of the medium was changed to 0.1% FBS, and incubation was continued until collection. Quiescent and senescent cells were continuously labelled for two weeks with BrdU (BrdU Labelling Reagent, Thermo Fisher) according to the manufacturer's protocol for labelling of culture cells. Cell were collected and counted; 5×10^5 cells were processed per condition. Genomic DNA was purified via phenol–chloroform extraction, RNase A-treated, and subsequently sheared using a Bioruptor UCD-200 instrument (pulse on low, 30 s on and 30 s off, 10 min total). DNA tubes were incubated in a heat block (100 °C) for exactly 1 min and then flash-frozen in liquid nitrogen. Tubes were let thaw at room temperature and 1 µg of purified anti-BrdU antibody (BD Pharmingen, 555627) was added per tube together with magnetic protein A/G beads and ChIP dilution buffer. Immuno-slurries were incubated overnight at 4 °C with constant rotation. Immuno-captured BrdU labelled DNA was purified according to the Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore, Sigma). Unbound DNA was kept as input. One microlitre of immunoprecipitated DNA was used in each qPCR reaction. Alternatively, to enrich for single-stranded BrdU-labelled DNA the heat-mediated denaturation was omitted and samples were processed for BrdU pull-down as above. The DNA second-strand was then generated by adding a mixture of random primers (Thermo Fisher), second-strand synthesis reaction buffer, dNTPs and DNA Pol I (New England Biolabs). The reaction was incubated for 4 h at 16 °C and subsequently purified by phenol–chloroform extraction. Following the second-strand synthesis, the double-stranded DNA was end-repaired with the End-It DNA End-Repair Kit (Epicentre, ER0720). Blunt-ended fragments were cloned using the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher), and then used to transform One Shot TOP10 chemically competent *Escherichia coli* (Thermo Fisher, C404010). Individual colonies were picked and subjected to Sanger sequencing using a T7 promoter primer at Beckman Coulter Genomics.

RNA-seq. Total RNA from early passage, early and deep senescent cells (Extended Data Fig. 1a) was extracted as described above. The total RNA was processed with the Illumina TruSeq Stranded Total RNA Ribo-Zero kit and subjected to Illumina HiSeq2500 2 × 125-bp paired-end sequencing using v4 chemistry at Beckman Coulter Genomics Inc. More than 70 million reads were obtained for each sample. The RNA-seq experiment was performed in three biological replicates.

Raw RNA-seq reads were aligned to the GrCh38 build of the human genome using HiSat2⁴². Counts mapping to the genome were determined using featureCounts⁴³. Counts were then normalized using the trimmed mean of M-values (TMM) method in EdgeR⁴⁴. EdgeR was additionally used to derive differential expression from the normalized dataset. Differential expression data were then ranked by log₂ fold change and input into the GenePattern interface for GSEA Preranked, using 1,000 permutations, to determine enrichment for KEGG pathways, SASP, and the interferon response^{45,46}. The outputs were then corrected for multiple comparisons by adjusting the nominal *P* value using the Benjamini–Hochberg method⁴⁷. Data were displayed using GENE-E software (<http://www.broadinstitute.org/cancer/software/GENE-E>).

In silico analysis of transcription factors binding to L1. Transcription factor profiles were created using ChIP-seq data from the ENCODE project (GEO accession numbers GSE2961 and GSE32465). Transcription factor ChIP-seq and input control reads were aligned to the consensus sequence of L1HS using bowtie1⁴⁸. The fold change, log₂(enrichment), was calculated per base pair of the L1HS consensus using the transcription factor ChIP-seq read coverage per million mapping reads (RPM) versus input control RPM values, and smoothed by LOESS smoothing with a parameter $\alpha = 0.1$. The total number of mapping reads used in RPM normalization was determined from a separate bowtie1 alignment to the human genome (hg19).

Construction of FOXA1 reporters. L1 promoter reporter plasmids L1WT and L1del (390–526) were obtained from S. Dmitriev^{49,50}. Both contain luciferase as the reporter cloned in the sense orientation. To determine antisense transcription from the same plasmid EYFP was inserted in the inverse orientation upstream of the L1 5' UTR as follows. The EYFP sequence was excised from pEYFP-N1 (Clontech, 6006-1) with AgeI and NotI and blunt ended. Plasmids L1WT and L1del were digested with XbaI, blunted and treated with FastAP (Fermentas). Successful insertion of anti-sense EYFP was verified using PCR primers AAAGTTCTTATGGCCGGGC (in EYFP) and GCTGAACCTTGTGGCCGTTTA (in L1 promoter) and Sanger sequencing. Plasmid pcDNA3.1/LacZ was used as the co-transfection control. Luciferase and β-galactosidase assays were performed as described⁴⁹. EYFP-N1 was used as a positive control for detecting the EYFP signal. Co-transfections were performed on early passage LF1 cells using Lipofectamine with Plus Reagent (Invitrogen) according to the manufacturer's instructions.

Lentiviral vectors. Constructs were obtained from public depositories as indicated below. Virions were produced and target cells were infected as described (<https://www.addgene.org/tools/protocols/plko/>). shRNA sequences were obtained from The RNAi Consortium (TRC, http://www.broad.mit.edu/genome_bio/trc/rnai.html), cloned into third-generation pLKO.1 vectors and tested for efficacy. Four selectable markers were used to allow multiple drug selections: pLKO.1 puro (2 µg ml⁻¹) and pLKO.1 hygro (200 µg ml⁻¹) (gifts from B. Weinberg, Addgene plasmid 8453, 24150), pLKO.1 blast (5 µg ml⁻¹) (gift from K. Mostov, Addgene plasmid 26655), and pLKO.1 neo (250 µg ml⁻¹) (gift from S. Stewart, Addgene plasmid 13425). pLKO-RB1-shRNA63 and pLKO-RB1-shRNA19 were gifts from T. Waldman (Addgene plasmids 25641 and 25640)⁵¹. For FOXA1 shRNAs, TRCN0000014881 (a) and TRCN0000014882 (b) were used. For TREX1 shRNAs, TRCN0000007902 (a) and TRCN0000011206 (b) were used. For knockdown of L1, we designed and tested nine shRNAs, of which two (shL1_11 to ORF1, AAGCAGTGTGTAGAGGGAAAT, and shL1_44 to ORF2, AAGACACATGCACACGTATGT) showed significant knockdown (Extended Data Fig. 5g) and were chosen for further work. The remaining seven shRNAs produced no or minimal knockdown. For CGAS shRNAs, TRCN0000128706 (a) and TRCN0000128310 (b) were used. For STING shRNAs, TRCN0000161345 (a) and TRCN0000135555 (b) were used.

All ectopic expression experiments used constructs generated by the ORFeome Collaboration (<http://www.orfeomecollaboration.org/>) in the lentivirus vector pLX304 (blestidicin resistant, Addgene plasmid 25890) and were obtained from the DNASU plasmid repository (<https://dnasu.org/DNASU/Home.do>): RB1 (ccsbBroad304_06846, HsCD00434323), TREX1 (ccsbBroad304_02667, HsCD00445909), FOXA1 (ccsbBroad304_06385, HsCD00441689).

All the interventions in senescent cells were initiated by infecting cells at 12 weeks of senescence (point D in Extended Data Fig. 1a). After appropriate drug selections, cells were incubated until 16 weeks of senescence (point E in Extended Data Fig. 1a), when they were collected for further analysis.

The 3× intervention was performed by infecting early passage LF1 cells sequentially with vectors pLKO.1 puro shRB, pLKO.1 hygro shTREX1 and pLX304 blast FOXA1 (Extended Data Fig. 5c). After each infection, the arising drug-resistant pool of cells was immediately infected with the next vector. After the third infection, the cells were collected for further analysis 48 h after the drug selection was complete. The infections were also performed in various combinations and in each case resulted in the activation of L1 expression, entry into senescence, and induction of an IFN-I response. The sequence above was chosen because it gave the most efficient selection of cells for further analysis. To allow an additional (fourth) intervention in 3× cells (shL1, shSTING or shCGAS), shRNAs targeting RB1 were recloned in pLKO.1 neo, thus freeing pLKO.1 puro for the fourth gene of interest. This allowed an efficient drug selection process and sample harvest 48 h after the last selection.

Retrotransposition reporters. The two-vector dual luciferase reporter system reported previously⁵² was adapted for lentiviral delivery. The L1_{RP}-FLuc reporters were recloned from plasmids pWA355 and pWA366 into the lentiviral backbone pLX304. pWA355 contains a functional, active L1_{RP} element, whereas pWA366 contains L1_{RP}(JM111), a mutated element carrying two missense mutations in ORF1 that is unable to retrotranspose. Early passage LF1 cells were infected with a puromycin-resistant lentivirus expressing RLuc. Pooled drug-resistant cells were then infected with high-titre particles of pLX304-WA355 or pLX304-WA366 constructs. Immediately after infection, cells were treated for four days with 3TC (at the indicated concentrations). Cells were then collected and assayed for RLuc and FLuc luciferase activities. The native L1 retrotransposition reporter pLD143⁵³ was co-transfected with pLKO vectors (shLuc, shL1_11 and shL1_44) into HeLa cells using FuGene HD (Promega). Cell culture, transfection and retrotransposition assays were done as described above. Retrotransposition activity was normalized to the activity of L1_{RP} co-transfected with shLuc. Three independent experiments were performed for each construct.

Identification of expressed L1 elements by long-range RT-PCR and 5' RACE. Total RNA was collected from cells using the Trizol reagent (Invitrogen). The RNA was further purified using the PureLink RNA Mini kit (Invitrogen) with DNase I digestion. From the eluted total RNA, poly(A) RNA was isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). The forward primer (MDL15UTRPRAF, primer set 1, Supplementary Table 1) was used with either of two reverse primers (MDL15UTRPRCR, primer set 3, amplicon size 537 bp) or MDL15UTRPRDR, primer set 4, amplicon size 654 bp). A high-fidelity thermostable reverse transcriptase (PyroScript RT-PCR Master Mix Kit, Lucigen) was used with 10 ng of poly(A) mRNA per reaction and amplified for 10 cycles. No template and RNaseA treated samples were used as negative controls. The generated amplicons were cloned into the TOPO-TA (Invitrogen) vector and the resulting plasmids were used to transform One Shot TOP10 chemically competent *E. coli*. Individual colonies were picked and subjected to Sanger sequencing using a T7 promoter sequencing primer at Beckman Coulter Genomics. A total

of 96 sequencing reactions (1 plate) were performed for each primer pair in four experiments for a total of 768 sequenced clones. Sequencing data were trimmed to remove the RT-PCR primers and BLASTed against the human genome (GRCh38) with a match/mismatch cost of +1, -4 and allowing species-specific repeats for *Homo sapiens*. Only perfect hits were scored and annotated for genomic coordinates. 658 clones could be mapped to the reference genome, 51 contained at least 1 mismatch and thus likely represent elements that are polymorphic in the cell line, and 58 were cloning artefacts. Whenever a clone presented multiple instances of perfect identity a fractional count was adopted, dividing the counts by the number of elements sharing the same sequence. Each mappable clone was further analysed using L1Xplorer⁵⁴ to recover the classification features of the L1 element and determine whether it is intact.

Alternatively, poly(A) RNA isolated as above was subjected to RACE. Each reaction contained 10 ng of poly(A) RNA and was processed using the 5'RACE System kit (Thermo Fisher, 18374-041). The two antisense gene-specific primers (GSP) used for 5'RACE were: for GSP1, MDL15UTRPRDR (primer set 4, Supplementary Table 1), and for the nested GSP2, MDL15UTRPRCR (primer set 3, Supplementary Table 1). Amplification products were cloned and sequenced as above, using a T7 promoter sequencing primer by Beckman Coulter Genomics. A total of 94 clones were sequenced; 26 contained mostly a polyG stretch generated by the tailing step in the RACE protocol and 18 could not be mapped to the human genome. The remaining 50 mappable clones contained L1 sequences and were aligned to the L1HS consensus using a setting of >95% identity at positions 1–450 (<http://www.girinst.org/repbase/update/browse.php>). The mappable clones were also assigned to individual L1 families using RepEnrich software⁵⁵. Pairwise alignments to the consensus were performed with LALIGN⁵⁶. Multiple sequence alignments were calculated using MAFFT (Multiple Alignment using Fast Fourier Transform) with the L-INS-i algorithm (accurate for alignments of <200 sequences)⁵⁷. Alignment visualization, percentage identity colouring and consensus were generated by Jalview⁵⁸.

Generation and analysis of CRISPR–Cas9 knockouts. Three distinct guide RNA (gRNA) sequences for each chain of the IFN α receptor (*IFNAR1* and *IFNAR2*), listed in the GeCKO v2.0 resource (F. Zhang laboratory, MIT; http://genome-engineering.org/gecko/?page_id=15)⁵⁹, were tested and the following ones were chosen: *IFNAR1* (HGLibA_29983) AACAGGAGCGATGAGTCTGTGA; *IFNAR2* (HGLibA_29985) GTGTATATCAGCCTCGTGT. Cas9 and gRNAs were delivered using a single lentivirus vector (LentiCRISPR_v2, Feng Zhang Laboratory, MIT; Addgene plasmid 52961), carrying a puromycin-resistance gene. The efficacy of the CRISPR–Cas9 mutagenesis, on the basis of which the above two gRNAs were chosen, was evaluated by treating the infected and drug-selected cells with interferon (universal type I interferon, PBL Assay Science, 11200-1) and monitoring nuclear translocation of phospho-STAT2 and IRF9 by immunofluorescence. The absence of translocation signifies lack of IFN-I responsiveness and hence loss of IFNAR function. Experimental procedures followed the protocols provided by the Zhang laboratory (<https://benchling.com/protocols/cNzMcO/cloning-customsgnas-into-crispr-lentiviral-vectors>)⁶⁰. In the experiments shown in Fig. 3d (replicative senescence) and Extended Data Fig. 5m, both *IFNAR1* and *IFNAR2* gRNAs were used to treat the same cells to further increase the efficacy of ablating the INF-I response. For early passage and senescent cells, co-infections of *IFNAR1* and *IFNAR2* vectors were performed followed by selection with puromycin. For senescent cells, high-titre lentivirus particles were applied to senescent cells at 12 weeks in senescence (point D, Extended Data Fig. 1a) and cells were assayed 4 weeks later (point E, Extended Data Fig. 1a). For the experiment shown in Fig. 3d (SIPS), edited early passage cells were single-cell cloned. 24 single cells were isolated using the CellRaft technology (Cell Microsystems) and expanded. Genomic screening of the CRISPR cut site was performed by the CRISPR Sequencing Service (CCIB DNA Core, Massachusetts General Hospital, <https://dnacore.mgh.harvard.edu/>). The successful knockout of *IFNAR1* and *IFNAR2* was verified in 4 out of the 24 expanded clonal cell lines.

Immunoblotting. Cells were collected in Laemmli sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT) and boiled for 5 min at 100°C. Whole-cell extracts (60 μ g protein) were separated by SDS–PAGE and transferred onto Immobilon-FL membranes (Millipore). Non-specific binding was blocked by incubation in 4% bovine serum albumin (BSA; Thermo Fisher) and 0.1% Tween-20 in PBS for 1 h at room temperature. Primary antibodies were diluted in the blocking solution and incubated overnight at 4 °C. A list of all the primary antibodies is provided in Supplementary Table 2. Secondary antibodies were diluted in blocking solution and incubated for 1 h at room temperature. Signals were detected using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). For the quantification of signals, all samples to be compared were run on the same gel. Loading standards were visualized on the same blot as the test samples using the LI-COR 2-colour system. Bands were imaged and quantified using LI-COR software. All bands to be compared were quantified on the same image and were within the linear range of detection of the instrument.

Immunofluorescence microscopy performed on cells in culture. Cells were grown on glass cover slips and the samples were processed as previously described⁶¹. Primary antibodies are listed in Supplementary Table 2. Staining of ssDNA was performed as described previously¹¹. In brief, cells seeded on coverslips were fixed on ice with 4% paraformaldehyde (PFA) for 20 min and then incubated in 100% methanol at -20 °C overnight. The cells were then treated with 200 mg ml⁻¹ RNase A at 37 °C for 4 h. Cells were blocked with 3% BSA and incubated overnight at 4 °C with primary antibodies diluted in 3% BSA. Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope or a Nikon Ti-S inverted fluorescence microscope. All microscope settings were set to collect images below saturation and were kept constant for all images taken in one experiment, as previously described⁶¹. Image analysis was performed as described below for tissues. **PCR arrays.** Total RNA was collected from cells as indicated above (see 'PCR' section) and analysed using the Qiagen RT² Profiler Human Type I Interferon Response PCR Array (PAHS-0162E-4). Reverse transcription reactions were performed with the Qiagen RT² First Strand Kit (330404) using 1 μ g of total RNA as starting material. Then, 102 μ l of the completed reaction was combined with 650 μ l of Qiagen RT² SYBR Green ROX qPCR Mastermix (330521) and 548 μ l of RNase-free molecular grade water, and run in the 384-well block on a Viia 7 Applied Biosystems instrument. All procedures followed the manufacturer's protocols. All conditions were run in triplicate. The results were analysed using the Qiagen GeneGlobe Data Analysis Center (<http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/>). In brief, C_t values were normalized to a panel of housekeeping genes. ΔC_t values were calculated between a gene of interest and the mean housekeeping genes value. Fold changes were then calculated using $2^{-\Delta\Delta C_t}$ formula. The lower limit of detection was set at C_t of 35. For any gene of interest to be considered significant, the following filters were set: (1) more than twofold change in expression; and (2) $P > 0.05$. In addition, genes with an average $C_t > 32$ in both control and test samples were also eliminated.

ELISA. IFN β levels were quantified with the VeriKine-HS Human IFN Beta Serum ELISA Kit (PBL Assay Science, 41415). Cell culture media were conditioned for 48 h before collection. To remove particles and debris, 1-ml aliquots were spun for 5 min at 5,000g. All incubations were performed in a closed chamber at room temperature (22–25 °C) keeping the plate away from temperature fluctuations. Next, 50 μ l of sample buffer followed by 50 μ l of diluted antibody solution were added to each well. Finally, 50 μ l of test samples, standards or blanks were added per well. Plates were sealed and shaken at 450 r.p.m. for 2 h. At the end of the incubation period, the contents of the plate were removed and the wells were washed three times with 300 μ l of diluted wash solution. Horseradish peroxidase (HRP) solution (100 μ l) was added to each well and incubated for 30 min under constant shaking. The wells were emptied and washed four times with wash solution. TMB substrate solution (100 μ l) was added to each well. Plates were incubated in the dark for 30 min. Finally, 100 μ l of stop solution was added to each well and within 5 min absorbance at 450 nm was recorded. The values recorded for the blank controls were subtracted from the standards as well as sample values to eliminate background. Optical density (OD) units were plotted using a 4-parameter fit for the standard curve and were used to calculate the interferon titres in the samples.

Human tissue specimens. Human skin specimens were collected as part of the Leiden Longevity Study^{62,63} and were provided by P.E.S. Informed consent was obtained and all protocols were approved by the ethical committee of the Leiden University Medical Centre. The samples were collected as 4-mm thickness full-depth punch biopsies, embedded in optimal cutting compound (OCT), flash-frozen, and stored at -80 °C. Samples were shipped on dry ice to Brown University. The Brown investigators were blinded to everything except the age and sex of the subjects. The OCT-embedded specimens were cryosectioned at 8- μ m thickness using a Leica CM3050S cryomicrotome. The slides were fixed with 4% PFA and 0.5% Triton X-100 in PBS (pre-warmed to 37 °C) for 20 min at room temperature. No further permeabilization was performed. Antibody incubation was preceded by a blocking step with 4% BSA (fraction V, Thermo Fisher), 2% donkey serum, 2% rabbit serum and 0.1% Triton X-100 in PBS for 1 h at room temperature. Primary antibodies were diluted in the above blocking solution (1:200) and incubated overnight at 4 °C with rocking in a humidified chamber. The secondary antibodies (AlexaFluor 546 and AlexaFluor 647, Life Technologies) were also diluted in blocking solution and incubated for 2 h at room temperature. Three 15-min washing steps in PBS, containing 0.2% Triton X-100, followed each antibody incubation. Nuclei were counterstained with 2 μ g ml⁻¹ DAPI in PBS, containing 0.2% Triton X-100, for 15 min. Stained slides were mounted with ProLong Antifade Mountant without DAPI (Life Technologies) and imaged on a Zeiss LSM 710 Confocal Laser Scanning Microscope. A z-series encompassing the full thickness of the tissue was collected for each field. All microscope settings and exposure times were set to collect images below saturation and were kept constant for all images taken in one experiment. Image analysis was performed using either CellProfiler software⁶⁴, or ImageJ open source software from the NIH (<http://rsbweb.nih.gov/ij/>).

Nuclei were defined using the DAPI channel. Cell outlines were defined by radially expanding the nuclear mask using the function 'Propagate' until an intensity threshold in the AlexaFluor 546 and AlexaFluor 647 channels was reached. The fluorescence intensity within these regions was then recorded in both channels. A total of 200 cells in several fields were scored for each condition. Mouse tissue sections were processed and analysed in the same way as described above.

Mouse tissue specimens. Total RNA was extracted from 50 mg of visceral adipose, small intestine, skeletal muscle, brown adipose or liver tissue by mincing followed by homogenization in Trizol (Invitrogen) using a Power Gen 125 homogenizer (Fischer Scientific). After phase separation, the RNA in the aqueous layer was purified using the Purelink RNA Mini kit (Invitrogen) with DNase I digestion. To assess gene expression by RT-qPCR 1 µg of total RNA was reverse transcribed as described above. In each individual experiment all samples were processed in parallel and no blinding was introduced.

Imaging of whole-mount white adipose tissue followed the method described previously⁶⁵. In brief, white adipose tissue (visceral depot) was subdivided into 0.5–1.0 cm³ sized pieces and incubated in 10 ml of fresh fixing buffer (1% PFA in PBS pH 7.4) for 30 min at room temperature with gentle rocking. After three washing steps with PBS, the tissue blocks were cut into six equal pieces. All subsequent incubations were performed in 2-ml cylindrical microcentrifuge tubes. Primary antibody incubation was preceded by a blocking step with 5% BSA and 0.1% saponin in PBS for 30 min at room temperature. Primary antibodies were diluted in the above blocking solution (1:200) and incubated overnight at 4 °C with gentle rocking. The secondary antibodies (AlexaFluor 546, AlexaFluor 594 and AlexaFluor 647, Life Technologies) were also diluted in blocking solution and incubated for 2 h at room temperature. Three 10-min washing steps in PBS followed each antibody incubation. Antibody-independent staining of nuclei and lipids was performed after immunostaining: DAPI and BODIPY (Thermo Fisher) were diluted in PBS with 5% BSA and incubated with tissue specimens for 20 min followed by three washing steps as above. Stained samples were carefully placed on confocal-imaging-optimized #1.5 borosilicate glass chamber slides. A small drop of PBS prevented drying. Acquired images were analysed as described above.

Co-staining of SA-β-Gal activity and ORF1 protein in liver sections was performed by staining for SA-β-Gal first as described⁶⁶. Subsequently, samples underwent heat-induced epitope retrieval by steaming for 20 min in antigen retrieval buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0). Samples were then processed for immunofluorescence staining as described in the 'Human tissue specimens' section.

Kidney tissue preserved in OCT was cryosectioned, treated for 10 min with 0.5% (w/v) periodic acid, then stained with periodic acid-Schiff's (PAS) reagent (Fisher Scientific, SS32-500) for 10 min. Stained tissue sections were mounted with Shandon Aqua Mount (Fisher Scientific, 14-390-5) and then imaged under bright-field illumination. Glomerulosclerosis was scored as described²⁶. In brief, 40 glomeruli per animal were assessed in a blinded fashion and assigned scores of 1–4: score of 1, <25% sclerosis; 2, 25–50% sclerosis; 3, 50–75% sclerosis; 4, >75% sclerosis. The feature used to assess sclerosis was the strength and pervasiveness of PAS-positive lesions within the glomeruli. As exemplified in Fig. 4e, a sclerotic glomerulus is more shrunken and stains more intensely with PAS.

Quadriceps muscles were embedded in OCT, sectioned at 12-µm thickness and mounted onto positively charged slides. Sections were stained with haematoxylin and eosin (H&E; (haematoxylin for 3 min, followed by 30 s in eosin). Mounted slides were imaged on a Zeiss Axiovert 200M microscope equipped with a Zeiss MRC5 colour camera. To measure muscle fibre diameter, the shortest distance across approximately 100 muscle fibres per animal was measured using ImageJ software as described²⁷.

Statistical treatments. Excel was used to perform general statistical analyses (such as mean, s.d. and *t*-tests). R software for statistical computing (64-bit version 3.3.2) was used for one-way ANOVA and Tukey's multiple comparisons post hoc test. For consistency of comparisons, significance in all figures is denoted as follows: **P* < 0.05, ***P* < 0.01. Sample sizes were based on previously published experiments and previous experience in which differences were observed. No statistical test was used to pre-determine sample size. No samples were excluded. All attempts at replication were successful. There were no findings that were not replicated or could not be reproduced. The nature and numbers of samples analysed (defined as *n*) in each experiment are listed in the figure legends. The number of independent experiments is also listed. The investigators were blinded when quantifying immunofluorescence results. Fields or sections of tissues for quantification were randomly selected and then scored, using methods as indicated for individual experiments. The investigators were also blinded when scoring glomerulosclerosis and muscle fibre diameter. For RNA-seq and PCR array experiments, the statistical treatments are described under those sections.

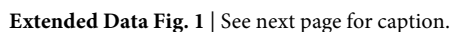
Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All Source Data and exact *P* values (if applicable) for every figure are included in the supporting information that accompanies the paper. RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) with accession number GSE109700. Any other data or information relevant to this study are available from the corresponding author upon reasonable request.

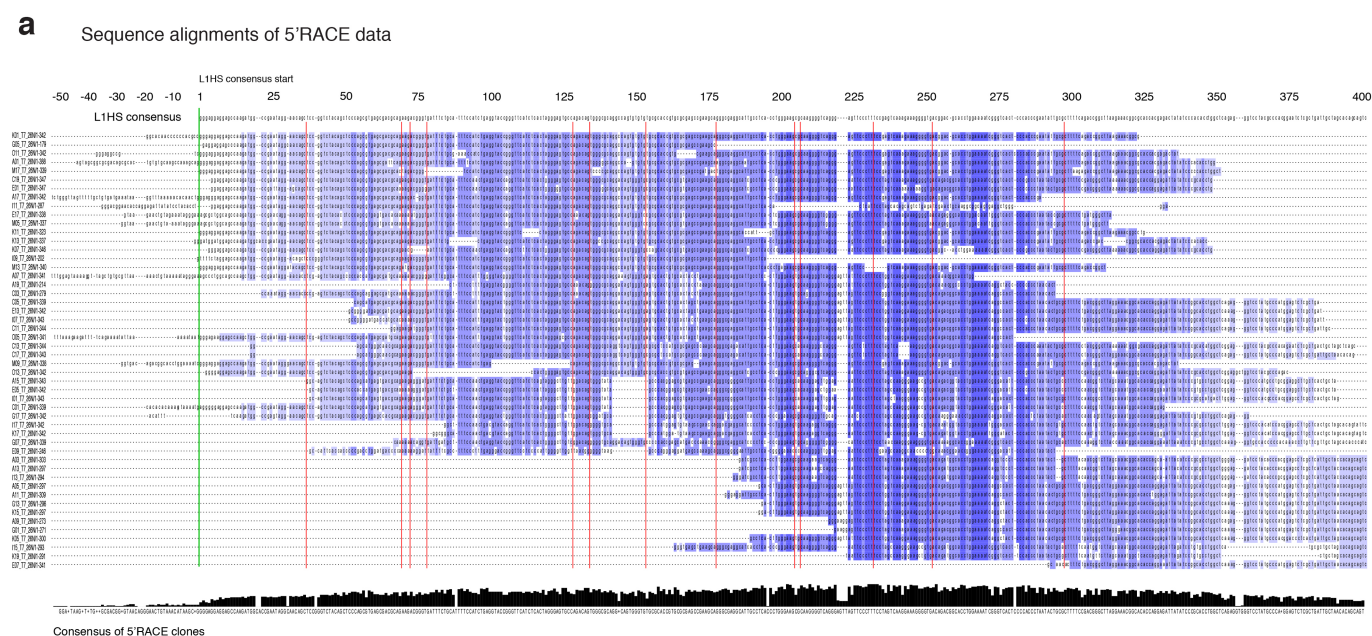
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Extended Data Fig. 1 | Establishment of senescent cultures and analysis of L1 and IFN-I activation. **a**, Passing regimen to obtain long-term replicatively senescent cells (details in Methods). Point 'A' was designated as zero for time in senescence. **b–d**, Confirmation of the senescent status of cultures. A representative experiment is shown; other experiments were monitored in the same manner and generated data that met these benchmarks. **b**, Cells were labelled with BrdU for 6 h. BrdU incorporation⁶⁷ and SA- β -Gal activity⁶⁶ were determined as indicated. DNA damage foci were visualized using γ -H2AX antibodies and immunofluorescence microscopy³⁴. **c**, Expression of p21 (CDKN1A) and p16 (CDKN2A) proteins was determined by immunoblotting. GAPDH was the loading control. For gel source data, see Supplementary Fig. 1. **d**, Expression of genes characteristic of the SASP was determined by RT-qPCR. **e**, L1 activation during senescence of IMR-90 and WI-38 strains of fibroblasts was assessed by RT-qPCR using poly(A)-purified RNA and primers for amplicon F (Fig. 1b). **f**, Long-range RT-PCR was performed with primers A (forward) and C (reverse) (amplicon G) and primers A (forward) and D (reverse) (amplicon H) (Fig. 1b, Supplementary Table 1) and the cDNAs were cloned and sequenced. Several attempts using the same protocol on early passage proliferating cells did not yield any L1 clones. Sequences were mapped to the unmasked reference genome demanding 100% identify. A total of 658 clones could be thus mapped, 51 additional clones contained at least 1 mismatch and

thus probably represent elements that are polymorphic in the cell line, and 58 were cloning artefacts. Among the 658 mappable clones, 224 unique elements were represented (Supplementary Table 3). Intact elements are the subset of full-length elements annotated with no ORF-inactivating mutations. Size of the features corresponds to the number of times the element was represented among the 658 clones. **g**, Summary of long-range PCR data presented in **f** and Supplementary Table 3. **h**, Apparent genomic copy numbers of elements detected with our amplicons (see Fig. 1b for locations of amplicons and Methods for primer design strategy). 'Predicted' denotes *in silico* PCR (Methods); 'observed' denotes qPCR was performed on 1 ng of genomic DNA and normalized to a known single copy locus. **i**, Activation of *IFNA* and *IFNB1* genes during senescence of WI-38 and IMR-90 cells was determined by RT-qPCR. **j**, Confirmation of the senescent status of cells in OIS (20 days, Fig. 1e) and SIPS (30 days, Fig. 1e) by SA- β -Gal activity. **k**, Confirmation of full-length L1 mRNA expression in all forms of senescence using RT-qPCR with primers for amplicons A and F on poly(A)-purified RNA. Late onset activation is shown by comparing days 9 and 20 for OIS, and days 12 and 30 for SIPS. $n = 3$ independent biological samples, repeated in two independent experiments (**b–e**, **i–k**). Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided *t*-test. Exact *P* values can be found in the accompanying Source Data.



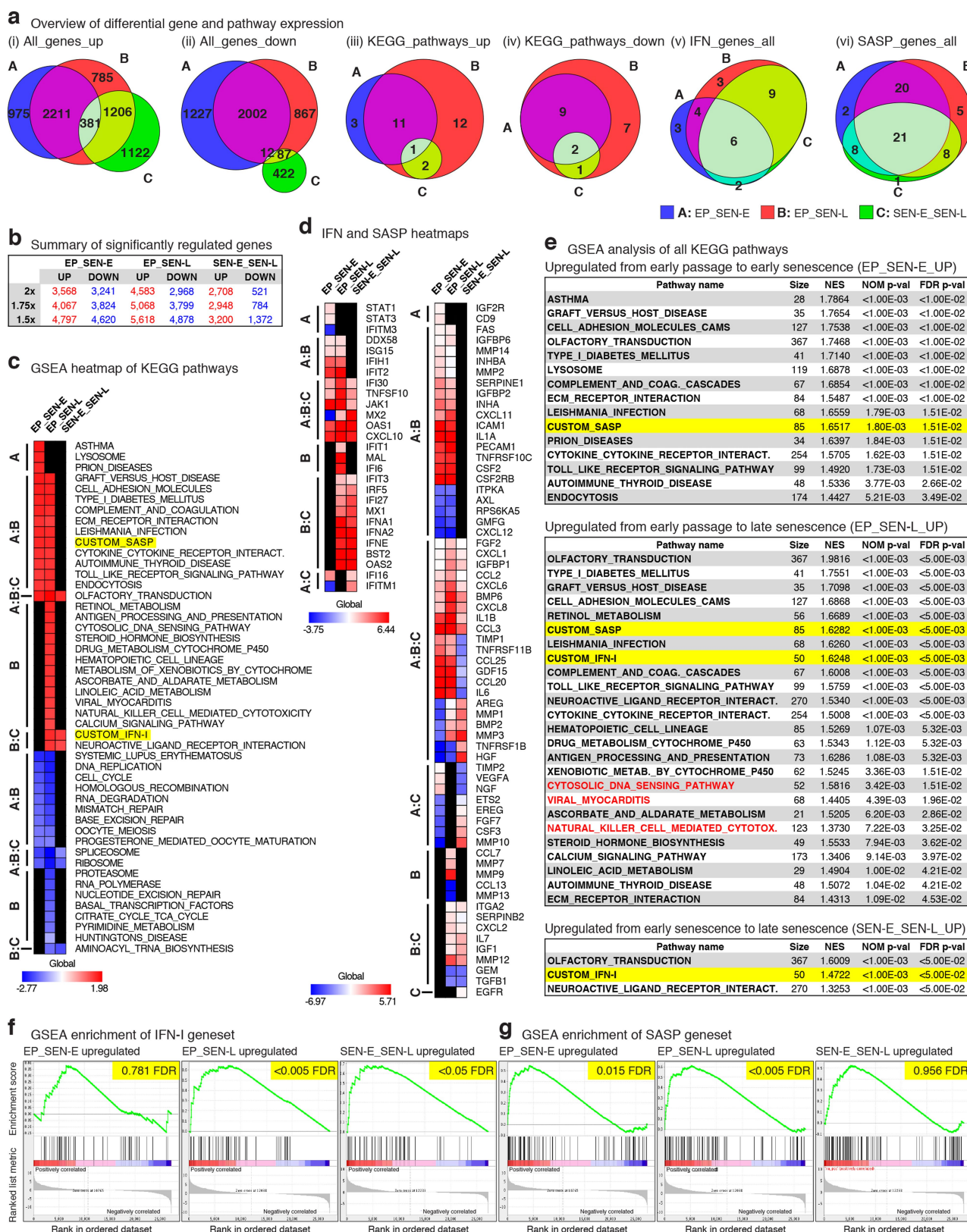
b Summary of 5'RACE data

Sequenced clones	50
Start: < -40 bp	4
-25 bp to +25 bp	28
+25 bp to +180 bp	6
> +180 bp	12

Family	Count	%
L1HS	8	16
L1PA2	9	18
L1PA3	11	22
L1PA4	7	14
L1PA5	8	18
L1PA7	2	4
L1P1	5	10

Extended Data Fig. 2 | Mapping transcriptional start sites in L1 elements activated during cellular senescence. 5' RACE was performed with primers C and D (Fig. 1a, Supplementary Table 1) on late senescent cells (16 weeks, point D in Extended Data Fig. 1a), the products were cloned, and individual clones were Sanger sequenced (Methods). **a**, A multiple sequence alignment of the 50 mappable clones against the L1HS consensus was generated with MAFFT software. The L1HS consensus is shown. Blue shading of the aligned clones shows their degree of identity with the consensus. Green vertical line marks the start (position 1) of the

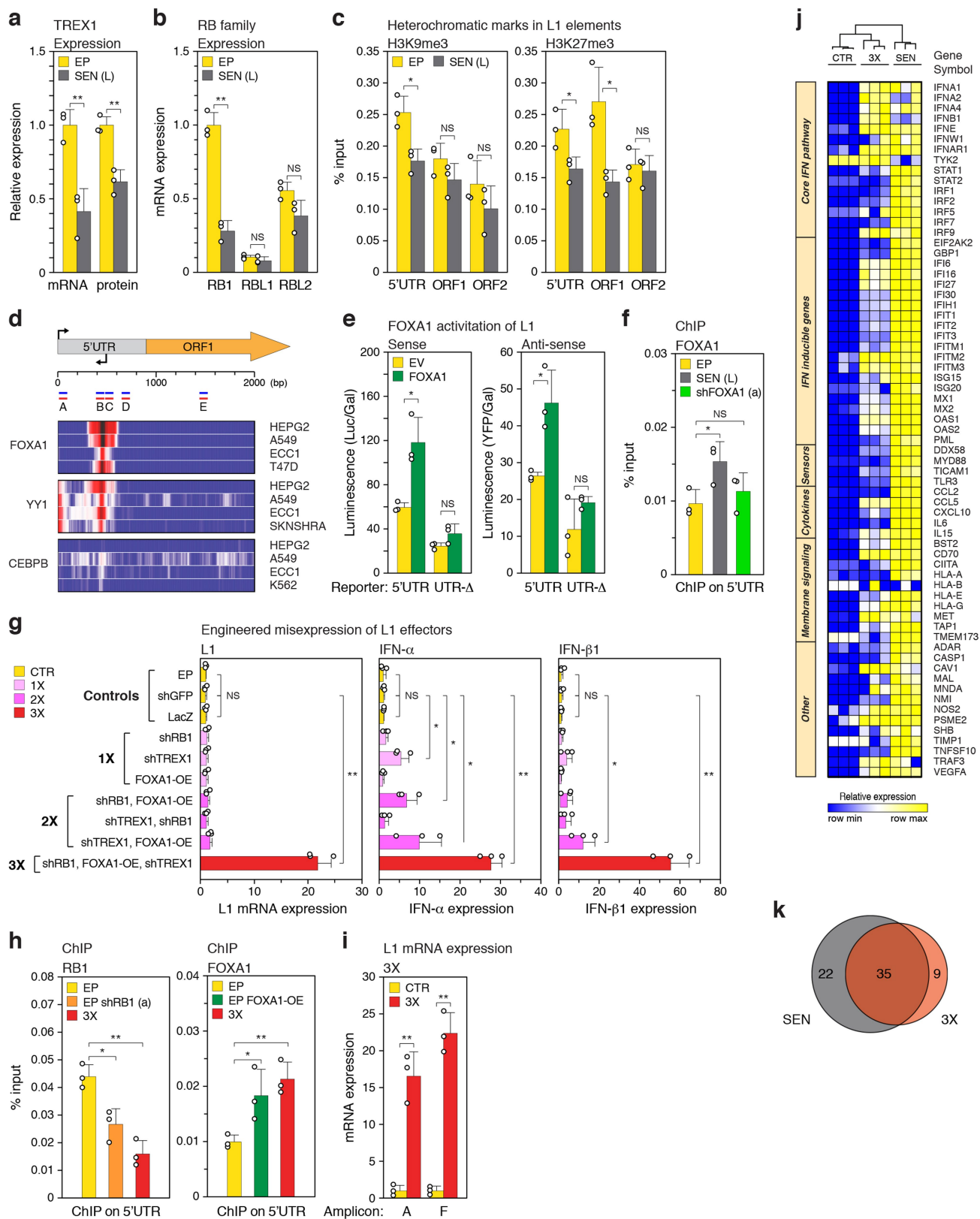
L1HS consensus. Red vertical lines mark short gaps (1–4 nucleotides) opened in the L1HS consensus by individual clones. The consensus of the 50 clones is shown at the bottom and was generated with Jalview. The initiation of L1 transcription is known to be imprecise, with most start sites occurring ± 50 bp of the consensus start site, and a subset as far down as +180 bp⁶⁸. **b**, Summary of the mapping data and classification of clones to families of L1 elements. The relative start sites were calculated relative to the L1HS consensus start site. RepEnrich software⁵⁵ was used to assign the clones to L1 families.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Evolution of transcriptomic changes during progression of cellular senescence. RNA-seq was performed on early proliferating LF1 cells and cultures at 8 weeks (SEN-E) and 16 weeks (SEN-L) in senescence (points C and D, respectively, in Extended Data Fig. 1a). Data were analysed using a three-way comparison: EP versus SEN-E, EP versus SEN-L and SEN-E versus SEN-L (see Methods for details). **a**, Area-proportional generalized Venn diagrams depicting the intersections of the three comparisons for the following datasets. **i–ii**, Significantly upregulated and downregulated genes (row '2×' in **b**). **iii–iv**, Significant KEGG pathways identified by gene set enrichment analysis (GSEA). Note the considerable evolution of the transcriptome in late senescence, exemplified by large changes (especially upregulated) in differentially expressed genes as well as pathways. **v–vi**, Significantly changing genes in the IFN-I and SASP gene sets (see Supplementary Table 4 for annotation of gene sets). Note that most changes in SASP genes occur early, whereas a large component of IFN-I changes is specific for late senescence. **b**, Summary of significantly changing genes using a fixed false discovery rate (FDR) (<0.05) and variable fold-change cut offs ($2\times$, $1.75\times$ and $1.5\times$). **c**, GSEA analysis of KEGG pathways. Heat map representation shows significantly upregulated pathways in red (also see **e**)

and downregulated pathways in blue. Non-significant comparisons are shown in black; vertical annotations refer to Venn diagrams in **a**, **iii–iv**. Note that the SASP gene set is upregulated early, whereas the IFN-I gene set is upregulated late. **d**, Heat maps of significantly changing genes in the IFN-I and SASP gene sets. Vertical annotations refer to Venn diagrams in **a**, **v–vi**. **e**, List of significantly upregulated KEGG pathways identified using GSEA (see Supplementary Table 5 for a list of all pathways). NES, normalized enrichment scores. IFN-I and SASP gene sets are highlighted in yellow. Note the significant upregulation of IFN-I between early and late senescence. Red font identifies KEGG pathways indicative of cytosolic DNA sensing and a type I interferon response at late times. **f**, **g**, GSEA profiles of the IFN-I and SASP gene sets for all comparisons; FDR is highlighted in yellow. Note that the upregulation of IFN-I is significant for EP_SEN-L and SEN-E_SEN-L but not for EP_SEN-E, and that the upregulation of SASP is significant for EP_SEN-E and EP_SEN-L but not SEN-E_SEN-L. $n = 3$ independent biological samples. Differential expression data were analysed for significance using the GSEA GenePattern interface and the outputs were corrected for multiple comparisons by adjusting the nominal P values using the Benjamini–Hochberg method (see Methods for details).

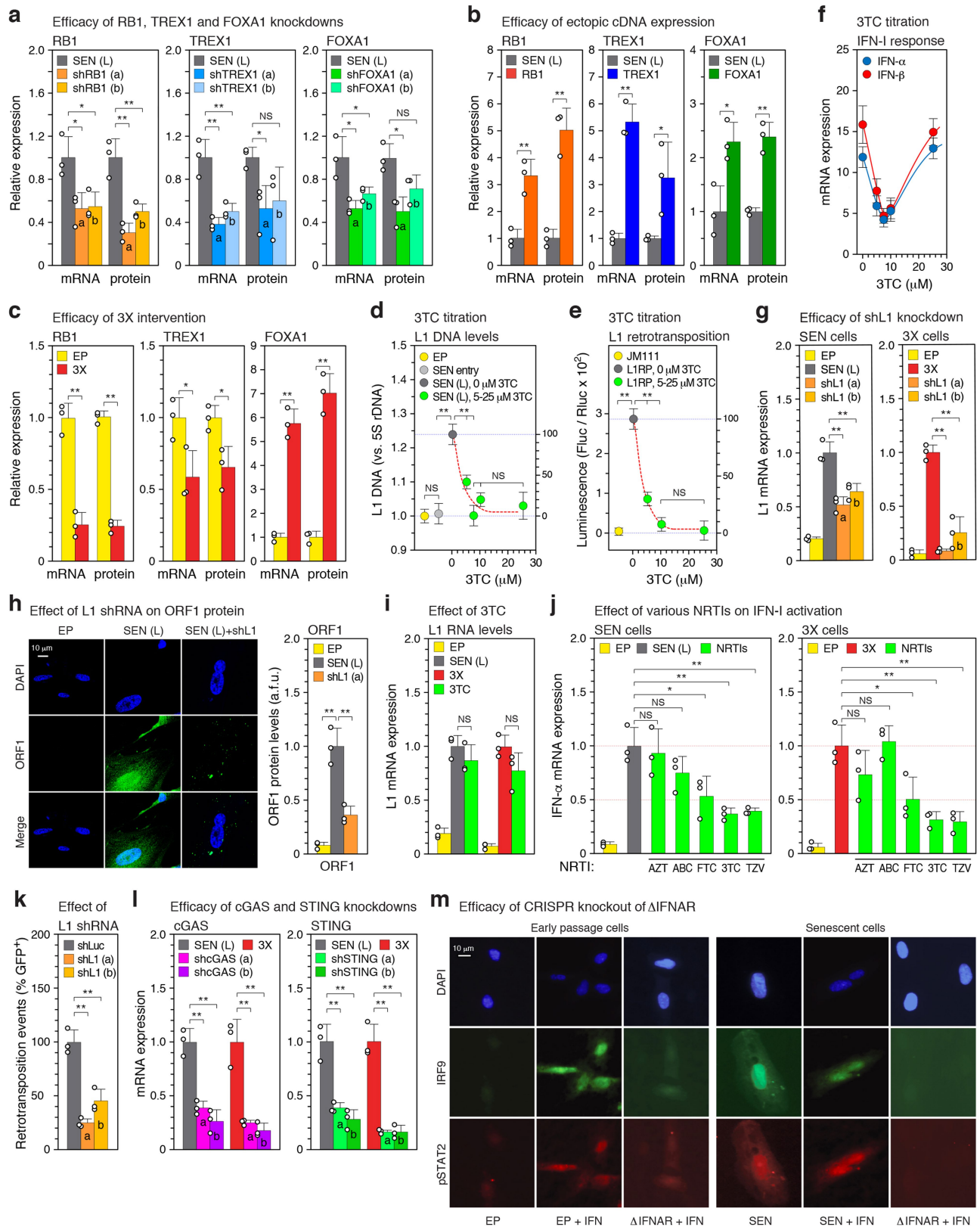


Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Characterization of L1 effectors and the IFN-I response. **a**, Expression of TREX1 was determined by RT-qPCR and immunoblotting. For gel source data, see Supplementary Fig. 1.

b, Expression of RB family genes was compared by RT-qPCR. Primer pairs for all genes were verified to be of equivalent efficiency. **c**, Enrichment of H3K9me3 and H3K27me3 on L1 elements was examined by ChIP-qPCR (PCR primers illustrated in Fig. 1b were used: 5' UTR, amplicon A; ORF1, amplicon E; ORF2, amplicon F). **d**, ChIP-seq data from ENCODE were investigated for transcription factors that bind to the L1 consensus sequence. The fold change, $\log_2$ (enrichment), relative to input controls is shown for the indicated cell lines. The binding of YY1 to the L1 promoter has been documented⁶⁹ and was used as a positive control. CEBPB was used as a negative control. A schematic illustrating L1 coordinates and relevant features is shown above. Amplicons A–E are the same as shown in Fig. 1b. **e**, Transcriptional activity of the intact L1 5' UTR or a UTR lacking the FOXA1-binding site (UTR- Δ) was determined using sense and antisense reporters cotransfected into early passage LF1 cells either with a FOXA1 expression plasmid or empty vector. **f**, FOXA1 was knocked down in senescent cells with shFOXA1 (**a**) (see also Fig. 2e and Extended Data Fig. 5a) and binding to the L1 5' UTR (amplicon B) was determined by ChIP-qPCR. **g**, Knockdown of RB1, TREX1 and ectopic expression of FOXA1 were performed in early passage cells in all single (1 $\times$), double (2 $\times$)

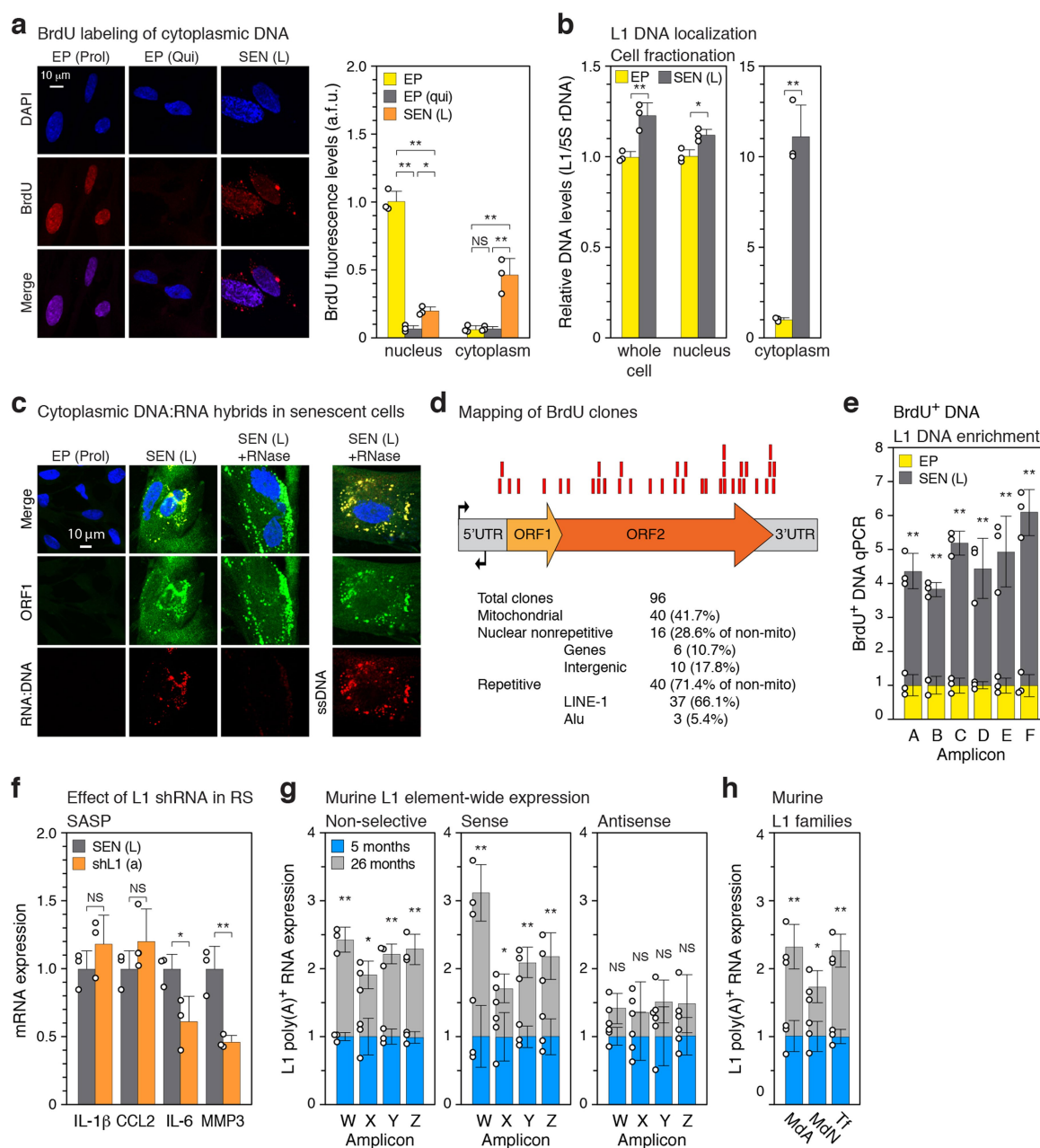
and triple (3 $\times$) combinations and assessed by RT-qPCR using poly(A)-purified RNA for activation of L1, *IFNA* and *IFNB1* expression (primers for amplicon F). Three controls are shown: cells infected with irrelevant shRNA (shGFP), expression construct (LacZ), or uninfected early passage cells. **h**, L1 5' UTR occupancy of RB1 and FOXA1 in 3 $\times$ cells was determined by ChIP-qPCR performed as in Fig. 2a, b. Primers for amplicons A and B were used for *RB1* and *FOXA1*, respectively. For comparison, single interventions in early passage cells with shRB1 (**a**) or *FOXA1* cDNA expression (EP FOXA1-OE) are also shown. **i**, Confirmation of full-length L1 mRNA expression in 3 $\times$ cells using RT-qPCR with primers for amplicons A and F on poly(A)-purified RNA. CTR, cells infected with irrelevant shRNA (shGFP). **j**, Heat map representation showing all biological replicates for the 67 genes significantly changing expression in SEN and/or 3 $\times$ cells (Fig. 2h, Supplementary Table 6). Column clustering was calculated as 1 – Pearson correlation. Rows have been grouped into functional subsets of the IFN-I response. **k**, Venn diagram showing the overlap between the 67 significantly changing genes. $n = 3$ independent biological samples, repeated in two independent experiments (**a–f**, **h**); $n = 3$ independent experiments (**g**, **i**.) Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided t -test. Exact P values can be found in the accompanying Source Data.



Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Efficacy of genetic and pharmacological interventions. **a, b**, Knockdowns with two distinct shRNAs (**a** or **b**) or ectopic cDNA expression (**b**) were performed in senescent cells as described in Fig. 2d, e, g (also see Methods). The effectiveness of these manipulations on their targets was assessed by RT-qPCR and immunoblotting. For gel source data, see Supplementary Fig. 1. **c**, RB1, TREX1 and FOXA1 mRNA and protein expression after the triple ($3\times$) intervention (Fig. 2f). **d**, The effect of 3TC treatment on the relative abundance of L1HS sequences in senescent cells was determined by multiplex TaqMan qPCR on total DNA (primer set 6, Supplementary Table 1). SEN entry, 0 weeks in senescence (Fig. 1a; point A in Extended Data Fig. 1a). 3TC was administered continuously from SEN entry until collection 16 weeks later. **e**, The dual luciferase L1 reporter system⁵² was used to determine the effect of 3TC dosing on retrotransposition. L1 reporters were introduced into early passage cells using lentivirus vectors (Methods) and cells were treated with 3TC for 4 days before collection and assay. JM111, a defective reporter carrying mutations in ORF1 (absence of 3TC); L1RP, a retrotransposition competent reporter. **f**, The effect of 3TC dosing on the IFN-I response. The experiment in **d** was processed by RT-qPCR to determine the expression of *IFNA* and *IFNB1*. **g**, Knockdowns of L1 elements were performed with two distinct shRNAs (**a** and **b**) in senescent cells (as in Fig. 2d, e, g) or $3\times$ cells (as in Fig. 2f). The effectiveness on L1 expression was assessed by RT-qPCR using poly(A)-purified RNA and primers F. **h**, Cells in the experiment in **g** were examined for levels of ORF1 protein by immunofluorescence. Image analysis was performed with CellProfiler software (Methods). More than 200 cells were examined for each condition (a.f.u., arbitrary fluorescence units). **i**, The L1 shRNA treatment in the experiment in **g** was substituted

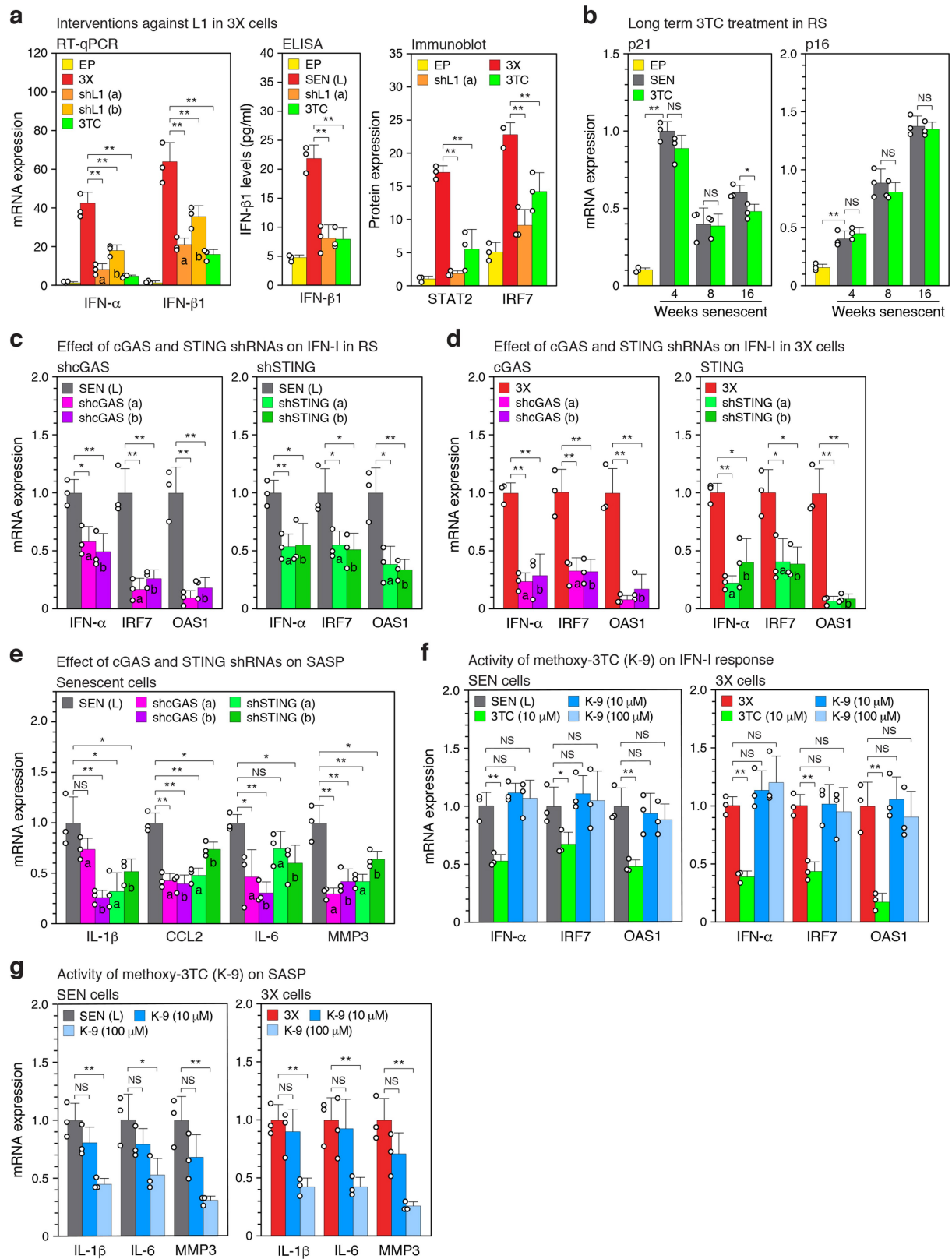
with 3TC treatment ($10\ \mu\text{M}$) for the same period of time. **j**, Five different NRTIs (or combinations) were tested for effects on the IFN-I response. AZT (zidovudine, $15\ \mu\text{M}$), ABC (abacavir, $15\ \mu\text{M}$), FTC (emtricitabine, $10\ \mu\text{M}$), 3TC ($10\ \mu\text{M}$) and TZV (Trizivir, a combination of $15\ \mu\text{M}$ AZT, $15\ \mu\text{M}$ ABC and $7.5\ \mu\text{M}$ 3TC). Cells were treated for 4 weeks between 12 and 16 weeks in senescence (Fig. 1a; points D and E in Extended Data Fig. 1a). $3\times$ cells (Fig. 2f) were treated with 3TC for 48 h after the completion of the last drug selection. *IFNA* expression was determined by RT-qPCR. **k**, A native L1 reporter (pLD143)⁵³ was co-transfected with shRNA plasmid vectors into HeLa cells (Methods). Retrotransposition was scored as GFP-positive cells, and shL1 knockdowns were normalized to a shLuc negative control. The absolute average retrotransposition frequency (percentage of GFP-positive cells) was 4.1, which matches the published values for the reporter used (pLD143)⁵³. **l**, Knockdowns of cGAS and STING were performed in senescent or $3\times$ cells as with the other shRNAs (as in **a**, **g**, and Fig. 2d, e, g). **m**, Downregulation of interferon signalling after CRISPR-mediated inactivation of *IFNAR1* and *IFNAR2* genes was verified by the absence of IRF9 nuclear translocation and STAT2 phosphorylation in response to interferon stimulation. Cells were infected with lentivirus vectors expressing Cas9 and gRNAs to both *IFNAR1* and *IFNAR2* (ΔIFNAR , Methods). After the infection, cells were reseeded on coverslips, treated with interferon for 2 h, and examined by immunofluorescence microscopy. The experiment was repeated three times with similar results. $n = 3$ independent experiments (**a–j**, **l**), $n = 3$ independent biological samples, repeated in two independent experiments (**k**). Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided *t*-test. Exact *P* values can be found in the accompanying Source Data.



Extended Data Fig. 6 | Characterization of cytoplasmic DNA in senescent cells.

a, Left, quiescent and senescent cells were treated with BrdU as in Fig. 3c and the cellular localization of BrdU incorporation was visualized by immunofluorescence microscopy. Proliferating cells (EP (Prol)) are shown as a positive control for nuclear BrdU incorporation. Right, the signals were quantified using CellProfiler software (Methods). More than 200 cells were examined for each condition. **b**, Senescent (and early passage control) cells (neither labelled with BrdU) were fractionated into nuclear and cytoplasmic fractions, and the representation of L1 sequences in these compartments (as well as whole cells) was assessed with qPCR as in Fig. 3c (TaqMan multiplex qPCR assay¹⁶, amplicon F, Fig. 1b). Note that the y-axis units differ by tenfold between the left and right panels. **c**, Cells were examined by immunofluorescence microscopy for the presence of ORF1 protein, RNA–DNA hybrids, and ssDNA. See Methods and Supplementary Table 2 for antibodies. The RNA–DNA signal in senescent cells largely colocalized with the ORF1 signal and was lost after RNase A treatment. The ssDNA signal also colocalized with the ORF1 signal and was exposed by RNase treatment. The experiment was repeated three times with similar results. **d**, The pulled-down BrdU-containing DNA (a and Fig. 3c; Methods) was cloned and Sanger sequenced. Of the 96 total clones examined, 37 mapped to L1. Red boxes represent the relative positions of these clones on the L1 consensus sequence. **e**, Senescent cells labelled with BrdU (a and Fig. 3c) were immunoprecipitated with

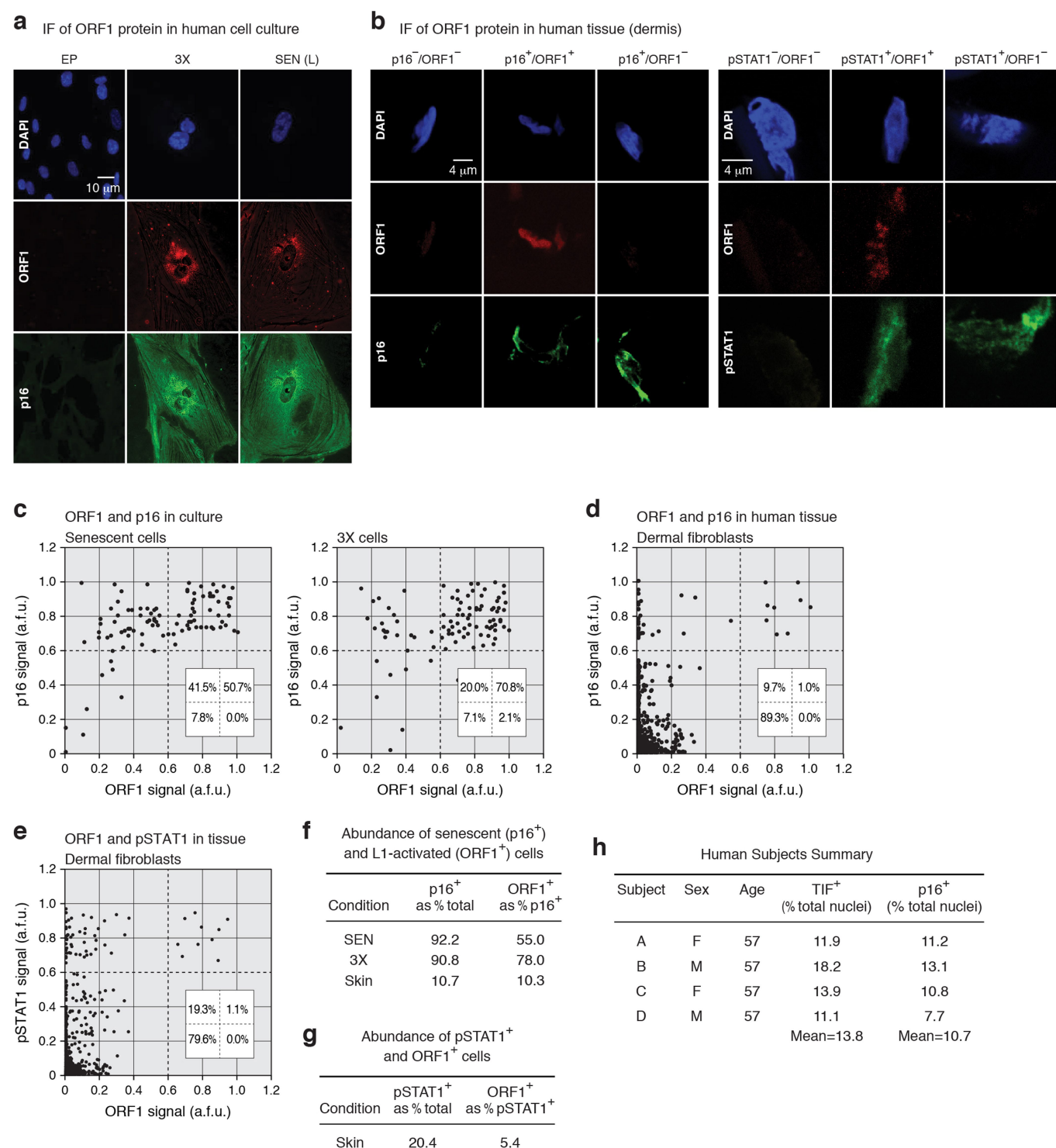
anti-BrdU antibodies, and the representation of L1 sequences in the pulled-down DNA was assessed using qPCR with primers spanning the entirety of L1 elements (Fig. 1b, c). **f**, Senescent cells were treated with L1 shRNA (using lentiviral vectors as described in Extended Data Fig. 5g) between 12 and 16 weeks of senescence, and expression of SASP genes was determined. **g**, Transcription throughout mouse L1 elements was assessed in a strand-specific manner using the same strategy as was applied to human L1 elements (Fig. 1b, c). The amplicons (designated W–Z to distinguish them from the human-specific primers) correspond to the 5' UTR (W), ORF1 (X), ORF2 (Y) and 3'UTR (Z). See Methods and Supplementary Table 1 for primer sequences (primer sets 37 and 48–50). Poly(A) RNA was prepared from male white adipose tissue. A total of 12 mice were assessed (3 pools of 4 mice each) in 3 independent experiments. **h**, Expression of the three currently active families of mouse L1 elements (Mda, MdN and Tf). Primers were designed to distinguish 5' UTR polymorphisms of the Mda, MdN and Tf families (Methods, Supplementary Table 1 primer sets 51–53). RT–qPCR was performed as in **f** (non-strand-specific). $n = 3$ independent biological samples, repeated in two independent experiments (**a**, **b**, **e**); $n = 3$ independent experiments (**f**). Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, one-way ANOVA with Tukey's multiple comparisons test (**a**), unpaired two-sided t -test (**b**, **e–h**). Exact P values can be found in the accompanying Source Data.



Extended Data Fig. 7 | See next page for caption.

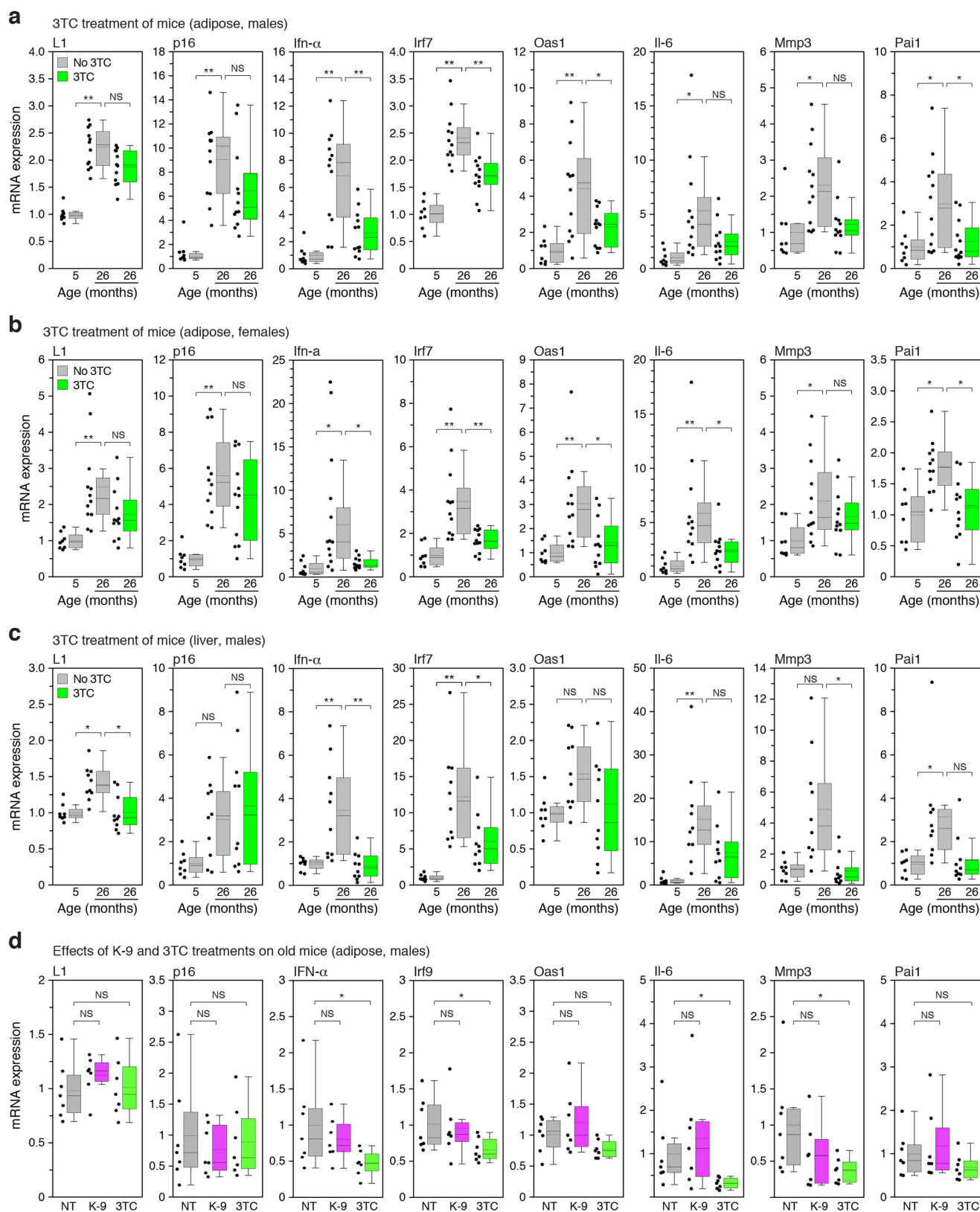
Extended Data Fig. 7 | Effects of ablating L1 activation, the cytoplasmic DNA sensing pathway, or interferon signalling on expression of the IFN-I and SASP responses. **a**, $3\times$ cells were treated with L1 shRNA or with 3TC for 48 h as described in Extended Data Fig. 5g,i. Effects on the IFN-I response were determined by RT-qPCR, ELISA or immunoblotting. For gel source data, see Supplementary Fig. 1. **b**, Cells were serially passaged into replicative senescence with 3TC (10 μ M) present throughout, as in Fig. 3f, and expression of the CDK inhibitors *p21* and *p16* was assessed by RT-qPCR. **c**, Senescent cells were treated with shRNAs against *CGAS* or *STING* between 12 and 16 weeks of senescence (as described in Extended Data Fig. 5l), and expression of IFN-I response genes (*IFNA*, *IRF7* and *OAS1*) was determined.

d, *cGAS* and *STING* knockdowns were performed with shRNAs in $3\times$ cells (as in **c**), and expression of IFN-I genes was examined by RT-qPCR. **e**, *cGAS* and *STING* were knocked down in senescent cells with shRNAs (as in **c**), and expression of SASP response genes (*IL1B*, *CCL2*, *IL6* and *MMP3*) was assayed by RT-qPCR. **f**, **g**, The activity of K-9 was compared with 3TC in senescent and $3\times$ cells. Senescent cultures were treated between 12 and 16 weeks (as in Fig. 3b) and $3\times$ cultures for 48 h (as in **a**). Effects on the expression of IFN-I genes (*IFNA*, *IRF7* and *OAS1*) and SASP genes (*IL1B*, *IL6* and *MMP3*) was assessed by RT-qPCR. $n = 3$ independent experiments. Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, unpaired two-sided *t*-test. Exact *P* values can be found in the accompanying Source Data.



Extended Data Fig. 8 | Assessment of p16, L1 ORF1 and pSTAT1 expression in senescent cells and skin specimens from aged humans. **a**, Immunofluorescence (IF) detection of p16 and ORF1 in early passage, 3X and senescent cells. **b**, Representative images of combinatorial ORF1 and p16 or ORF1 and phosphorylated STAT1 (pSTAT1) staining in human dermis. The experiments in **a** and **b** were repeated three times independently with similar results. **c**, Cells were plated on coverslips, stained and quantified as described in the Methods. A total of 200 cells in several fields were scored for each condition. Insets show the percentage of cells found in each quadrant. **d**, **e**, Abundance of ORF1 and p16 or pSTAT1 cells in human skin. Skin biopsies were cryosectioned and stained as described in the Methods. A total of 200 dermal fibroblast cells in

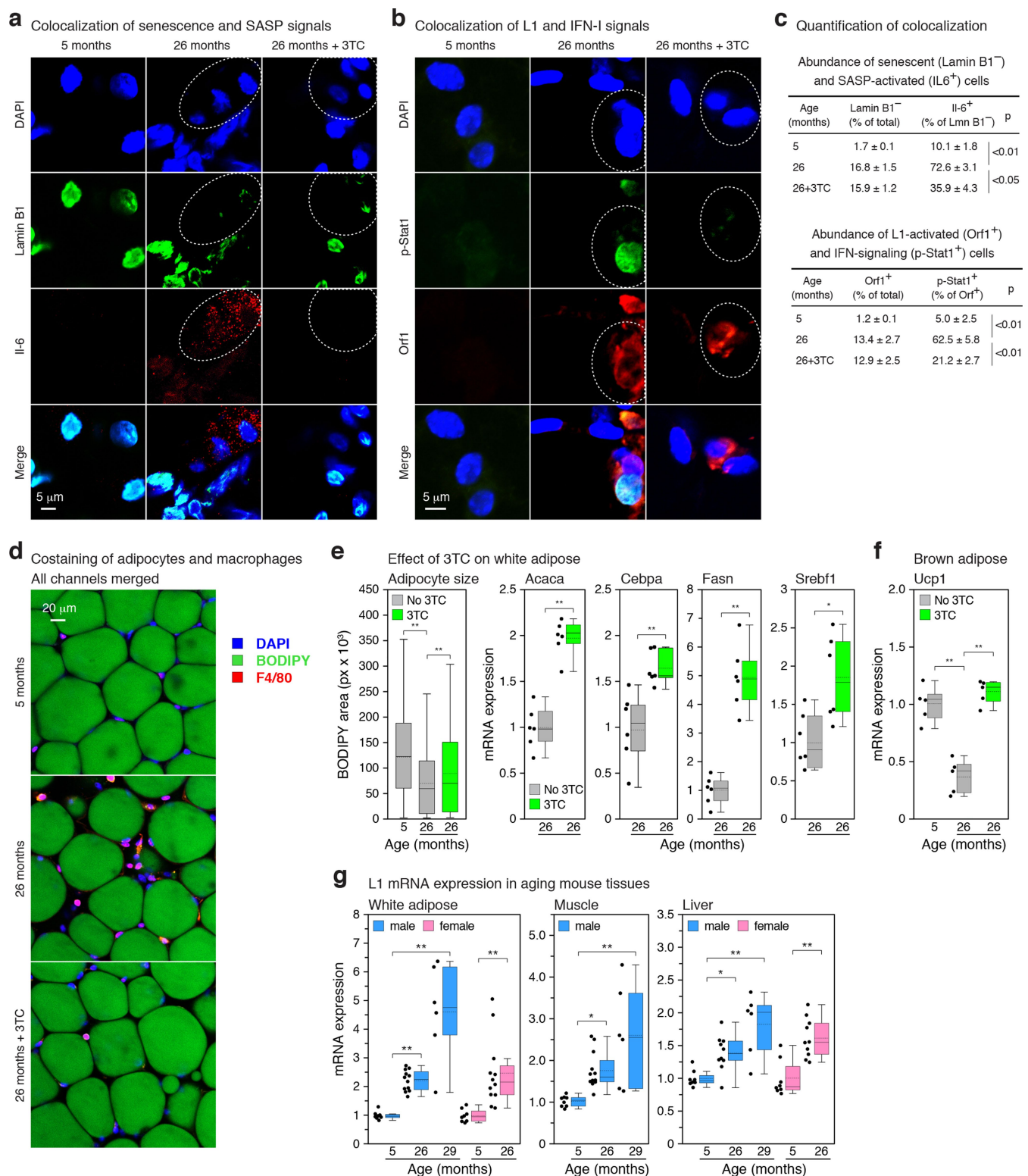
several fields were scored for each subject. Aggregated data for 4 subjects (800 cells) are shown. **f**, Data in **c** and **d** were recalculated to show the relative abundance of p16⁺ cells among all cells, and ORF1⁺ cells in the p16⁺ pool of cells. **g**, Data in **e** were recalculated as in **f**. **h**, Characteristics of the human subjects used in the analysis of dermal fibroblasts. These specimens were collected as part of the ongoing Leiden Longevity Study⁶². The specimens used here were chosen randomly from leftover material. The telomere dysfunction-induced foci (TIF) assay³⁴ relies on a two-parameter (colour) visualization of telomeres (using a FISH probe) and immunofluorescent detection of DNA damage foci (using antibody to 53BP1). Because of limiting material, it was not possible to combine detection of p16 with TIFs in a three-colour experiment.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Effects of 3TC or K-9 treatment on L1, p16, IFN-I and SASP gene expression in mouse tissues. **a–c,** Mice at the indicated ages were treated with 3TC continuously for two weeks (see also Fig. 4c, e, Extended Data Fig. 10d–f and Methods). For all conditions, the expression of L1 mRNA, *p16*, three representative IFN-I response genes (*Ifna*, *Irf7* and *Oas1*) and three representative SASP genes (*Il6*, *Mmp3* and *Pai1*) were assessed by RT-qPCR. In no instance was expression at 5 months plus 3TC significantly different from the no-drug control, therefore these data are not shown in the figure (for all collected data, see Supplementary Table 7). Each point represents one animal. **a,** Visceral white adipose, male mice. $n = 8$ mice at 5 month; $n = 12$ mice at

26 months; $n = 12$ mice at 26 months + 3TC. **b,** Visceral white adipose, female mice. $n = 8$ mice at 5 months; $n = 12$ mice at 26 months; $n = 12$ mice at 26 months + 3TC. **c,** Liver, male mice. $n = 8$ mice at 5 months; $n = 10$ mice at 26 months; $n = 10$ mice at 26 months + 3TC. **d,** Mice at the age of 26 months were treated with K-9 or 3TC in drinking water for two weeks and analysed by RT-qPCR as above. NT, not treated. Visceral white adipose, male mice, $n = 7$ mice for each group. Box plots as in Fig. 4. Data are mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, one-way ANOVA with Tukey's multiple comparisons test. Exact P values can be found in the accompanying Source Data.



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Combinatorial assessment of senescence, IFN-I, SASP and L1 markers and effects of 3TC on age-associated phenotypes in mouse tissues. **a, b,** Whole-mount immunofluorescence was performed on white adipose of 5- and 26-month-old male mice (with and without 2 weeks of 3TC treatment). **a,** Loss of lamin B1 (senescence marker) was colocalized with IL-6 (SASP marker). **b,** pSTAT1 (IFN-I marker) was colocalized with ORF1 (L1 marker). **c,** Quantification of the experiments in **a** and **b.** Four mice and at least 200 cells per animal were scored for each condition. **d,** Neutral lipids were stained with BODIPY to visualize mature adipocytes in whole-mount preparations, and macrophages were detected by immunofluorescence using the F4/80 antibody. **e,** The effects of 2 weeks of 3TC treatment on adipogenesis were assessed by measuring mean adipocyte size (left), and by RT-qPCR to determine the expression

of key adipogenic genes (right; *Acaca*, acetyl-CoA carboxylase 1; *Cebpa*, CCAAT/enhancer-binding protein alpha; *Fasn*, fatty acid synthase; *Srebf1*, sterol regulatory element-binding protein 1). Adipocyte size (BODIPY-stained area) was calculated using CellProfiler; aggregated data for 5 mice and 500 total cells are shown. For RT-qPCR data, each point represents one animal; $n = 6$ mice. **f,** Expression of the *Ucp1* gene (thermogenin) in brown adipose tissue was determined by RT-qPCR and is represented as in **e.** $n = 5$ mice. **g,** Expression of L1 mRNA was determined by RT-qPCR and is represented as in **e.** $n = 8$ mice at 5 months; $n = 12$ mice at 26 months; $n = 6$ mice at 29 months. Box plots as in Fig. 4. Data are mean $\pm$ s.d. $*P \leq 0.05$, $**P \leq 0.01$, one-way ANOVA with Tukey's multiple comparisons test (**c, e, left, f, g**), or unpaired two-sided *t*-test (**e, right**). Exact *P* values can be found in the accompanying Source Data.

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Statistical parameters

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n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
- ☐ ☒ Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about [availability of computer code](#)

Data collection

Collection of qPCR data: ExpressionSuite Software by Applied Biosystems, version 1.2.3.
Microscope imaging: confocal, ZEN Black Edition SP1 by Carl Zeiss, version 8.1. Fluorescence, NIS Elements by Nikon, version 4.50. Brightfield, AxioVision by Carl Zeiss, version 4.8.2 SP3.
Immunoblot Imaging: Image Studio by LI-COR Biosciences, version 5.2.
ELISA plate reader: SoftMax® Pro software by Molecular Devices, version 5.0.1.
Luciferase reporter assays: Modulus Fluorometer by Turner Biosystems, version 1.3

Data analysis

Excel was used to perform general statistical analyses (means, s.d., t-tests, etc).
 For 1-way ANOVA and Tukey's post-hoc test R software for statistical computing (64-bit version 3.3.2) was used.
 For RNAseq data analyses, the following software were used: Bowtie, HiSat2, featureCounts, EdgeR, RepEnrich and the GenePattern interface for GSEA Preranked.
 To detect and annotate L1 elements the web front-end L1Xplorer was used.
 GeneGlobe Data Analysis by Qiagen was used to analyze PCR array data.
 CellProfiler (version 2.1.1) by Broad Institute was used for image analysis.
 ImageJ (version 1.50i) was used for western blot quantification.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Sequencing data that support the findings of this study are available in GEO with the primary accession code GSE109700. All source data and exact P values (if applicable) for every figure are included in the supporting information that accompanies the paper.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The nature and size of all samples are described in figure legends for all experiments. Sample sizes used were based on previously published experiments and experience from the Sedivy lab. No statistical test was used to pre-determine sample size.
Data exclusions	No pre-set criterion or data exclusion was used.
Replication	Results shown are representative of several independently performed experiments. Number of biological replicates and independent experiments is described in figure legends. All attempts at replication were successful. There were no findings that were not replicated or could not be reproduced.
Randomization	Genotype of C57BL/6 mice is known to investigators. No pre-established selection criteria for mice were used, other than gender and ages. When mice with desired gender were at appropriate ages, all mice in corresponding cages were used. No selection criteria were applied. Animals were assigned randomly to cohorts (drug-treated, control) by a technician that was blinded to the appearance or other characteristics of the animals.
Blinding	The investigators were blinded when quantifying IF results. Fields or sections of tissues for quantification were randomly selected and scored, as indicated in Methods. The investigators were also blinded when scoring glomerulosclerosis and muscle fiber diameter.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☒ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about [availability of materials](#)

Obtaining unique materials

Kamuvudine-9 (Ref. Ambati, J., Fowler, B. & Ambati, K. Compositions and Methods for Treating Retinal Degradation. PCT Patent Publication WO/2016/138425 (2016)) is available upon reasonable request from Dr. J. Ambati.

Antibodies

Antibodies used

Immunoblotting – GAPDH – (Cell Signaling Technology, Rabbit, cat.no. 5174, clone: n/a, 1:5000)
 Immunoblotting – GAPDH – (Sigma, Mouse, cat.no. G8795, clone: n/a, 1:5000)
 Immunoblotting – p16 – (Santa Cruz, Mouse, cat.no. sc-1661, clone: F-12, 1:1000)
 Immunoblotting – p21 – (Santa Cruz, Rabbit, cat.no. sc-756, clone: H-164, 1:1000)
 Immunoblotting, ChIP – RB1 – (BD Biosciences, Mouse, cat.no. 554136, clone: G3-245, 1:1000 WB, 1:50 ChIP)
 Immunoblotting – TREX1 – (Cell Signaling Technology, Rabbit, cat.no. 12215, clone: n/a, 1:1000)
 Immunoblotting – FOXA1 – (Abcam, Rabbit, cat.no. ab170933, clone: EPR10881, 1:1000)
 Immunoblotting – STAT2 – (Cell Signaling Technologies, Rabbit, cat.no. 4594, clone: n/a, 1:1000)
 Immunoblotting – IRF7 – (Abcam, Rabbit, cat.no. ab109255, clone: EPR4718, 1:1000)
 ChIP – FOXA1 – (Abcam, Goat, cat.no. ab5089, clone: n/a, 1:50)
 Immunofluorescence – p16 – (Santa Cruz, Mouse, cat.no. sc-56330, clone: JC8, 1:100)
 Immunofluorescence – Phospho-STAT1 – (Santa Cruz, Mouse, cat.no. sc-8394, clone: A-2, 1:50)
 Immunofluorescence – Phospho-STAT2 – (Millipore, Rabbit, cat.no. 07-224, clone: n/a, 1:50)
 Immunofluorescence – IRF9 – (Novus Biologicals, Rabbit, cat.no. NBP2-16991, clone: n/a, 1:100)
 Immunofluorescence, Immunoprecipitation – BrdU – (BD Biosciences, Mouse, cat.no. 555627, clone: 3D4, 1:100)
 Immunofluorescence – p-H2A.X – (Millipore, Mouse, cat.no. 05-636, clone: JBW301, 1:100)
 Immunofluorescence – F4/80 – (Abcam, Rat, cat.no. ab6640, clone: Cl:A3-1, 1:200)
 Immunofluorescence – ssDNA – (Enzo, Mouse, cat.no. ALX-804-192, clone: F7-26, 1:100)
 Immunofluorescence – DNA:RNA – (Kerafast, Mouse, cat.no. ENH001, clone: S9.6, 1:100)
 Immunofluorescence – LaminB1 – (Santa Cruz, Goat, cat.no. sc-6216, clone: C-20, 1:200)
 Immunofluorescence – IL-6 – (Cell Signaling Technologies, Rabbit, cat.no. 12912, clone: D5W4V, 1:200)
 Immunofluorescence – Human LINE-1 ORF1 – (Gift of K.H. Burns, Johns Hopkins)
 Immunofluorescence – Mouse LINE-1 Orf1 – (J.D. Boeke, abEA02, RabMAb clone: NYU-2-1_2)

Validation

With the exception of LINE-1 ORF1 antibodies, all antibodies are from commercially available sources and have been validated from the manufacturer with supporting publications found on manufacturer websites. Antibodies were further validated in-house using relevant positive and negative controls.

The human LINE-1 ORF1 antibody was validated in the lab of K.H. Burns, Johns Hopkins, as described in Rodic et al. Am. J. Pathol. 184, 1280-6 (2014).

The mouse LINE-1 Orf1 antibody was developed and validated in the lab of J.D. Boeke by transfection experiments and peptide blocking.

See below for summary of commercially available antibodies:

Immunoblot – p16 – Santa Cruz, sc-1661

Species: Human, Mouse, Rat

Application: ELISA, Immunocytochemistry, Immunofluorescence, Immunohistochemistry, Western Blot

Immunoblot – p21 – Santa Cruz, sc-756

Species: Mouse, Rat and Human

Application: ELISA, Immunoprecipitation, Immunofluorescence, Western Blot

Immunoblot, ChIP – RB1 – BD Biosciences, 554136

Species: Human, Mouse, Rat, Monkey, Quail, Mink (Reported)

Application: Western blot, Intracellular staining (flow cytometry), Bioimaging, Immunohistochemistry-formalin, Immunoprecipitation, Immunohistochemistry-frozen

Immunoblot – TREX1 – Cell Signaling Technologies, 12215

Species: Human

Application: Western blot

Immunoblot – FOXA1 – Abcam, ab170933

Species: Human, Mouse, Rat

Application: Western blot, Intracellular staining (flow cytometry), Bioimaging, Immunohistochemistry-formalin, Immunohistochemistry-frozen

Immunoblot – STAT2 – Cell Signaling Technologies, 4594

Species: Human

Application: Western blot

Immunoblot – IRF7 – Abcam, ab109255

Species: Human, Mouse, Rat

Application: Western blot, Intracellular staining (flow cytometry), Immunofluorescence, Immunohistochemistry-formalin, Immunoprecipitation, Immunohistochemistry-frozen

ChIP – FOXA1 – Abcam, ab5089

Species: Human, Mouse

Application: ChIP, Western blot, Immunohistochemistry-formalin, Immunoprecipitation

Immunofluorescence – p16 – Santa Cruz, sc-56330

Species: Human

Application: Western blot, Immunofluorescence, Immunohistochemistry, Immunoprecipitation, solid-phase ELISA

Immunofluorescence – p-STAT1 – Santa Cruz, sc-8394

Species: Human, Mouse and Rat

Application: Western blot, Immunofluorescence, Immunoprecipitation, Flow-cytometry, solid-phase ELISA

Immunofluorescence – p-STAT2 – Millipore, 07-224

Species: Human, Mouse

Application: Western blot, Immunofluorescence, Immunoprecipitation

Immunofluorescence – IRF9 – Novus Biologicals, NBP2-16991

Species: Human, Monkey

Application: Western blot, Immunofluorescence, solid-phase ELISA, Immunohistochemistry

Immunofluorescence, Immunoprecipitation – BrdU – BD Biosciences, 555627

Species: Human

Application: Flow cytometry, Immunofluorescence, Immunoprecipitation, Immunohistochemistry

Immunofluorescence – IRF9 – Novus Biologicals, NBP2-16991

Species: Human, Monkey

Application: Western blot, Immunofluorescence, solid-phase ELISA, Immunohistochemistry

Immunofluorescence - p-H2A.X – Millipore, 05-636

Species: Vertebrates

Application: Immunocytochemistry, Immunofluorescence, Western Blot, ChIP, Immunohistochemistry

Immunofluorescence – F4/80 – Abcam, ab6640

Species: Mouse

Application: Immunocytochemistry, Immunofluorescence, Western Blot, Radiomunoassay, Immunohistochemistry

Immunofluorescence – ssDNA – Enzo Life Sciences, F7-26

Species: Species Independent

Application: Flow Cytometry, Immunocytochemistry, Immunofluorescence, Immunohistochemistry

Immunofluorescence – RNA:DNA – Kerafast, S9.6

Species: Species Independent

Application: Dot Blot, Affinity Binding Assay, ChIP, Immunocytochemistry, Immunofluorescence, Immunohistochemistry, Immunoprecipitation

Immunofluorescence – Lamin B1 – Santa Cruz, sc-6216

Species: Human, Mouse and Rat

Application: Western Blot, Immunoprecipitation, Immunofluorescence, Immunohistochemistry, solid-phase ELISA

Immunofluorescence – IL-6 – Cell Signaling Technologies, 12912

Species: Mouse

Application: Western Blot, Immunoprecipitation, Immunofluorescence, Flow cytometry

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	LF1 cells were derived from embryonic lung tissue as described (Brown et al., Science, 1997). These cells have been in continuous use in our laboratory since their isolation in 1996. IMR-90 and WI-38 cells were obtained from the ATCC. HeLa cells were also obtained from the ATCC. 293T cells were only used for lentiviral packaging, and were obtained from Clontech.
Authentication	LF1 fibroblasts were authenticated by in-depth genome-wide sequencing analyses. IMR-90, WI-38, HeLa, and 293T cell lines were obtained from the sources above and used at low passage. They were not further authenticated.
Mycoplasma contamination	All cell cultures were periodically tested for mycoplasma. All cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	None of these cell lines are listed in the International Cell Line Authentication Committee (ICLAC) database.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	C57BL/6 mice were purchased from the Aged Rodent Colonies operated by the National Institute on Aging. Mice of both sexes were obtained at 5 and 18 months of age. The 5 month old animals were sacrificed after a short (1 week) acclimatization period. The 18 month old animals were housed until they reached a desired age. Mice were fed ad libitum on a regular diet, and handled following institutional regulations and guidelines. Both sexes were included in the study.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve field-collected samples.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics	Human skin specimens were collected as part of the Leiden Longevity Study (Schoenmaker, M. et al., Eur. J. Hum. Genet., 2006) and were provided by P. Eline Slagboom, Leiden University Medical Centre, Netherlands. 420 families of Caucasian origin took part in the Leiden Longevity Study (LLS). This group consisted of 991 long-lived brothers and/or sisters, their children and partners of their children. The samples were collected as 4 mm thickness full depth punch biopsies, embedded in optimal cutting compound (OCT), flash frozen, and stored at -80°C. Samples were shipped on dry ice to Brown University. The Brown investigators were blinded to everything except the age and sex of the subjects
Recruitment	The men were considered to be long-lived if they were 89 years or older, and the women if they were 91 years or older. Families were invited to participate when there were at least two long-lived brothers and/or sisters alive. Informed consent was obtained and all protocols were approved by the ethical committee of the Leiden University Medical Centre. The results of this study are purely observational and are not affected by any bias in cohort recruitment.



Sex-specific aging in animals: Perspective and future directions

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 Award Number: 2021305

Abstract

Sex differences in aging occur in many animal species, and they include sex differences in lifespan, in the onset and progression of age-associated decline, and in physiological and molecular markers of aging. Sex differences in aging vary greatly across the animal kingdom. For example, there are species with longer-lived females, species where males live longer, and species lacking sex differences in lifespan. The underlying causes of sex differences in aging remain mostly unknown. Currently, we do not understand the molecular drivers of sex differences in aging, or whether they are related to the accepted hallmarks or pillars of aging or linked to other well-characterized processes. In particular, understanding the role of sex-determination mechanisms and sex differences in aging is relatively understudied. Here, we take a comparative, interdisciplinary approach to explore various hypotheses about how sex differences in aging arise. We discuss genomic, morphological, and environmental differences

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between the sexes and how these relate to sex differences in aging. Finally, we present some suggestions for future research in this area and provide recommendations for promising experimental designs.

KEYWORDS

aging, comparative biology, lifespan, mortality, sex differences

1 | INTRODUCTION

Aging (or senescence) is the decline or deterioration in physiological, biochemical, and physical function seen with increased age. At the population scale, such deterioration in organismal phenotypes often manifests as declining fertility and increasing mortality with advancing adult age. It is well known that animal reproductive aging can differ between the sexes (reviewed in Comizzoli & Ottinger, 2021), with an extreme example being reproductive cessation in some female, but not male mammals (Alberts et al., 2013) with a long post-reproductive lifespan being seen in human females (Levitis et al., 2013). Remarkably, sex differences in lifespan are observed in many animal species as well and can encompass differences between males and females in the age-of-onset of senescence, the rate of increase in age-specific mortality, and/or the initial mortality rate in early adulthood—all of which can lead to sex-specific aging trajectories. Although aging is a near universal phenomenon, sex differences in aging are varied throughout the animal kingdom, with some species showing extreme sex differences and others showing none (Austad & Fischer, 2016). For example, in the ant *Lasius niger*, female queens live up to 28 years, female workers live several years, and males typically die within 2–3 months (Jemielity et al., 2007). In contrast, species lacking sex differences in lifespan are well documented among a variety of different species groups, including many mammals (Austad & Fischer, 2016) and birds (Liker & Szekely, 2005). Currently, the causes of this diversity in sex differences in aging across the animal kingdom are not well understood and present a fascinating problem for comparative biologists.

To study sex differences in aging and lifespan requires an understanding of how senescence evolves, as well as its genetic and environmental components. The evolutionary genetics of senescence is the subject of ongoing investigation, particularly in wild-dwelling populations where senescence evolved [reviewed in (Bronikowski & Promislow, 2005)]. Aging likely arises due to age-specific mutation–selection balance. If selection declines with advancing age [a null expectation in populations that have young-skewed age distributions (Hamilton, 1966), see Box 1], two genetic processes may occur. Mutations with deleterious late-age phenotypes may accumulate across generations (Medawar, 1946) and/or antagonistically pleiotropic mutations may accumulate with positive fitness effects in early life and negative fitness effects in late life (Williams, 1957). If age-specific selection differs between the sexes—for example, through sex-specific sources and drivers of mortality, then sex-specific senescence can evolve provided genetic variation exists. Such sexually

dimorphic selection trajectories can arise through sex-specific age distribution skews, sex-specific responses to the environment, or sex-specific habitats and behaviors (reviewed in Lemaître et al., 2020). As an extreme example, consider systems in which males compete for female mates with combat or bright ornamentation. It has been shown elsewhere that such behavioral and morphological differences between the sexes can increase male mortality relative to female mortality. Such a difference would give rise to different age structures between males and females, from which sex-specific senescence can evolve (e.g., Kappeler, 2017; Schacht et al., 2017). A less extreme example is seen in sex differences in resistance to infectious disease, particularly during pregnancy when female mammals can be more susceptible to infection [as seen in Soay sheep (Leivesley et al., 2019)]. Here too, high mortality in females could give rise to sexual dimorphism in age structure, and concomitant sexual dimorphism in mutation–selection balance.

Intimately entwined with sex-specific selection is the mechanism of sex determination (Hägg & Jylhävä, 2021). Determining the evolutionary genetic dynamics and environmental contributors to age-specific senescence from mechanisms of sex determination—sex chromosomes (heteromorphic, homomorphic, ancient, new, absent, etc.) and sex-determining loci—remains a major challenge in the understanding of the evolution of sex-specific aging. For example, an early driver of sexual differentiation once sex is “determined” is the reproductive steroid hormones (androgens and estrogens/progestins). Reproductive hormones are important in early sexual dimorphism in addition to their primary role in sexual maturation (reviewed in de Vries et al., 2014). But in oviparous TSD reptiles, maternally allocated steroid hormones in the yolk can also mediate sex determination itself (Bowden & Paitz, 2021). Disentangling the role of sex chromosomes, sex-determining loci, and hormonally mediated sexual development is difficult, but there has been good progress in specific model organisms for this endeavor. For example, in the Four Core Genotype mice, these factors are decoupled, and work on this model has revealed potential independent contributions from both factors (Davis et al., 2019). Interestingly, nature has provided additional model organisms whose sex-determination mechanism recommends them for addressing this question. For example, closely related species pairs that have genotypic sex determination versus environmental sex determination can reveal the role of sex-specific genetic loci versus hormonal cascades in the development of sex-specific aging (e.g., in reptiles, see turtle data in Box 1). At the mechanistic level, determining whether and which proximate (molecular) mechanisms of senescence have diverged between the sexes is a

BOX 1 Age-structured populations and selection

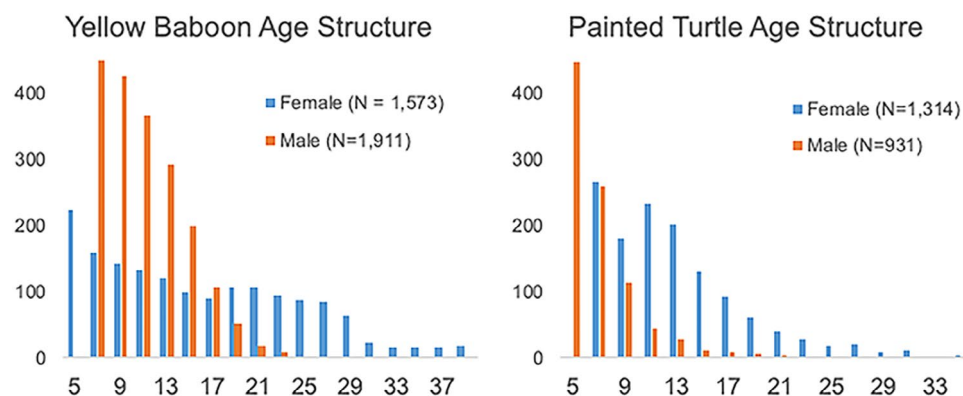


FIGURE 1 Sex-specific age structure of adult wild baboons (*Papio cynocephalus*) and painted turtles (*Chrysemys picta*). Data from Bronikowski et al. 2011 and Bronikowski et al. 2021, respectively. In both populations, the male distributions are left-skewed relative to the female distributions, and females have right extended distributions. The intensity of selection acting against a mutation that decreases age-specific survival declines more rapidly with age for males in both species. Baboons have genotypic sex determination (degenerate sex chromosome in males). Painted turtles have environmental (temperature) sex determination (no sex chromosomes).

As Medawar noted (see text), senescence evolves in senescent-free populations if age-structure decreases with advancing age classes (for example, age-independent predation or accidents that accumulate with number of years alive). This skew causes the strength of selection (i.e., the sensitivity of fitness to a small change in age-specific survival) to decline with age (see Charlesworth, 1992). That is, the effect of a change in age-specific survival early in life would have a much greater effect on population growth rate (r from the Euler-Lotka equation) than an equal change later in life. The role of fertility is such that in species where fertility also declines with advancing age, the decline exacerbates the declining intensity of selection. Whereas, in species that exhibit increasing fertility with age (such as in turtles), an active area of study is whether such increasing fertility can offset the declines in selection intensity due to declining numbers of older individuals (See box 1 in Promislow and Bronikowski, 2006). The development of the formal mathematical theory for the evolution of senescence is attributed to Hamilton (1966). A full description of these considerations and theory can be found in Charlesworth (1992). Baboons and painted turtles were chosen to highlight the differences in sex-determination mechanism (see Figure 1). Particularly interesting is the case of species with environmental sex determination, where genomic content and architecture are presumably identical between the sexes at fertilization.

complementary challenge. To this end, we convened a series of workshops in October 2020, bringing together experts from a wide range of biological disciplines to tackle these questions. Here, we report on the ideas, questions, and challenges identified. Our emphases were genomic architecture differences between the sexes, including those deriving from sex-determining mechanisms in contributing to sex-specific aging and longevity.

1.1 | Sex differences in aging in humans

Life expectancy of human females, on average, exceeds that of males across different human populations and historical times for which data are available (Mauvais-Jarvis et al., 2020; Sampathkumar et al., 2020). This pattern is observed also in several other primate species (Bronikowski et al., 2011; Colchero et al., 2016). The longest recorded lifespan for women is 122 years, whereas for men it is 116 years.

This difference in lifespan leads to marked female bias in the sex ratio of older cohorts. For example, the age pyramid of the United States shows the typical slight male bias in the youngest age group (0–4 years), then equivalency between males and females, until a bias toward females starts to appear at approximately 50 years of age (US Census Bureau). This female bias becomes most extreme in the oldest age group, 85 years or older, which includes more than twice as many women than men. This bias is also evident in the probability of reaching the age of 85, which is ~36% for men but ~50% for women based on the US Social Security Administration's Period Life Table for 2019. This shift with age toward an increased fraction of women among the surviving individuals is a common characteristic of human populations and illustrates how sex differences in aging impact population structure. By uncovering the mechanisms by which sex-specific aging occurs in other organisms, we will better understand onset of disease and progression in the aging human population, which could include how we might reverse or slow this process.



In addition to the well-documented sex differences in human lifespan, there are many other sex differences in how humans senesce (Austad, 2006; Austad & Fischer, 2016; Sampathkumar et al., 2020). These differences can be seen both at the organismal level as well as in various molecular measures. On the organismal level, while having an overall longer lifespan, women tend to be more “frail” and suffer more from physical ailments than men as they age (Collard et al., 2012; Gordon & Hubbard, 2020; Gordon et al., 2017; Marck et al., 2016). Osteoporosis, for example, is four times more common in women than in men. Women are also more prone than men to suffer from dementia and other age-associated neurological diseases. For instance, women have approximately twice the risk of developing Alzheimer's disease than men (Ferretti et al., 2018). This increased level of frailty and physical impairment in women is partially due to their longer lifespan, but research suggests that other factors play a role as well, including genetics and possibly hormone biology (Ferretti et al., 2018). Despite this increased frailty seen in women, they die at lower rates than men from 13 of 15 top causes of death in the US (Austad & Fischer, 2016). On the molecular level, men and women show differences in how their immune response changes with age (Klein & Flanagan, 2016), and women tend to show fewer somatic mutations and chromosomal abnormalities than men with age [reviewed in (Fischer & Riddle, 2018)]. However, the molecular events that lead to the observed organismal level sex differences in human aging are not well understood.

1.2 | Sex differences in aging across animal diversity

Like humans and many non-human primates, a number of species exhibit female-biased lifespans and slower aging (Austad & Fischer, 2016), yet numerous other species exhibit male-biased lifespan and slower aging, and still others lack sex differences in aging entirely (Figure 2). Among mammals, females tend to have longer lifespans than males, but exceptions do exist. For example, lifespan is equivalent for both sexes in American beavers (Clutton-Brock & Isvaran, 2007), and in some bat species, including Brandt's bat, males are longer lived than females (Kowalski et al., 2002; Podlitsky et al., 2005). In wild roe deer, which often show a female survival advantage, the increase in lifespan for females over males ranges from 0% to 30% depending on environmental factors (Garratt et al., 2015). This within-class variation in longevity has been observed broadly across vertebrates, including birds, reptiles, amphibians, and fish (Tower & Arbeitman, 2009; Xirocostas et al., 2020). Similarly, insects and other invertebrates show striking variation in sex-specific longevity. Social insects offer some of the most extreme examples, though are complicated by the fact that often two or more types of females exist with very different lifespans [i.e., worker and queen bees (Vaiserman, 2014; Xirocostas et al., 2020)]. Yet sex differences in aging are also known from other diverse insect taxa, including both hemimetabolous and holometabolous lineages (Bilde et al., 2009; Song et al., 2017; Zajitschek et al., 2009). Differences

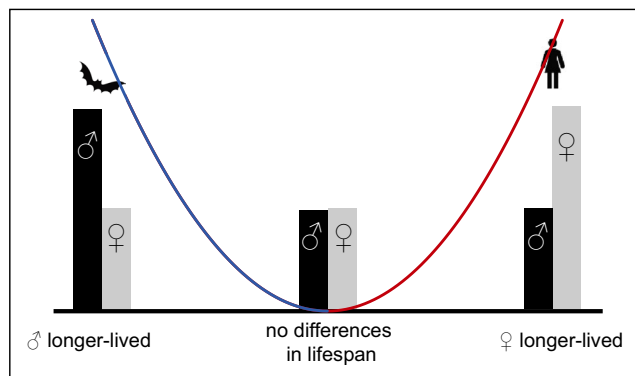


FIGURE 2 Sex differences in lifespan vary widely across animal taxa. Gray and black bars represent lifespan in females and males. Humans are an example of a species where females live longer, while in Brandt's bat, males live longer. See text for more examples. The curve illustrates that sex differences in lifespan (absolute value of $\text{lifespan}(f) - \text{lifespan}(m)$) form a continuum, from males living longer shown (left) to females living longer (right)

in lifespan between sexes or reproductive modes may be extreme in aquatic invertebrates as well; females of the rotifer *Brachionus manjavacas* live twice as long as males, and fertilized sexual females live 25% longer than asexual females (Snell, 2014). Sex differences in lifespan also vary widely in dioecious and androdioecious nematode worms, and again, outcomes seem to vary substantially depending on rearing conditions (Ancell & Pires-daSilva, 2017). These examples illustrate that sex differences in terms of lifespan are widespread and highly variable across the animal tree of life.

Other aspects of senescence also show sex differences in diverse animal taxa. For example, reproductive potential declines with age at different rates in males and females of many species (Comizzoli & Ottinger, 2021; Holmes et al., 2001). In the red wolf, *Canis rufus*, male reproductive success, as measured by pup recruitment, declines with age, while no such decline is observed in females (Sparkman et al., 2017). This situation is reversed in the black-footed ferret (*Mustela nigripes*) with females showing decreasing fertility after 3 years of age, while male fertility drops later, at 6–7 years of age (Wolf et al., 2000). Further, on the molecular level, we see that sexes can differ in the rate of decrease of telomere length (Barrett & Richardson, 2011; Gardner et al., 2014). In some species, males with a shorter lifespan have shorter telomeres earlier in life than females, as is the case in humans and many laboratory rodents (Barrett & Richardson, 2011; Gardner et al., 2014). There are also species such as ants and gulls where there is neither a relationship between telomere length and lifespan nor a sex difference in the telomere decay rate (Fischer & Riddle, 2018). For example, in two species of long-lived bats, no relationship between telomere length and age was detected in either sex (Foley et al., 2020; Lorenzini et al., 2009; Power et al., 2021), and a recent meta-analysis of 51 taxa found no consistent sex differences in telomere length (Remot et al., 2020). The examples of reproductive senescence and telomere length degradation illustrate the tremendous variation across animal taxa in the organismal and molecular features that show sex differences with aging.



1.3 | Mechanisms leading to sex differences in aging

In 2013, Lopez-Otin and colleagues proposed nine “hallmarks of aging,” that is, features that are commonly seen in aging animals across a wide range of species (López-Otín et al., 2013; for a similar characterization of seven “pillars of aging” see Kennedy et al., 2014). These shared characteristics of aging include “genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication” (López-Otín et al., 2013, see Box 2). The relationships among these molecular hallmarks, and their relationship to senescence—either causal or consequential—remain unknown outside of a few model species (i.e., humans, mouse, fruit flies).

Research has revealed a variety of pathways contributing to aging, as summarized in the discussion of the nine hallmarks of aging (López-Otín et al., 2013; but see Gems & de Magalhães, 2021). The molecular basis of sex differences in some species and absence of these differences in other species is not well defined, currently. Sex differences in any of the molecular drivers leading to the hallmarks of aging, or other biological mechanisms absent from the hallmarks, could potentially be involved. For example, males and females might have different levels of DNA repair enzymes, leading to different efficiencies in DNA damage repair and genome instability, thus impacting aging. This might be the case in *Drosophila*, as overexpression of DNA repair genes can impact males and females differently (Shaposhnikov et al., 2015). Similarly, males and females might differ in the rate at which they produce or clear senescent cells, thus leading to different rates at which senescent cells accumulate in various tissues. Sex differences in aging could also involve tissue-specific pathophysiological mechanisms affecting specific organs, including sex-specific organs, and resulting in age-related pathologies (for an example of this in *Drosophila*, see Regan et al., 2016). While some data from model organisms exist for some of the molecular drivers (Fischer & Riddle, 2018; Menees et al., 2021; Mitchell et al., 2016; Rodriguez-Fernandez et al., 2020; Tsurumi & Li, 2020), comprehensive investigations across taxa are lacking. Thus, the molecular pathways involved in generating sex differences in aging could be diverse and are poorly understood.

While sex differences in some molecular pathways associated with aging have been documented (reviewed in Hägg & Jylhävä, 2021; see also Baar et al., 2016; Brown-Borg et al., 1996; Hwangbo et al., 2004; Selman et al., 2008; Svensson et al., 2011; Yoon et al., 1990) the triggers of these differences remain obscure (but see Chen et al., 2012; Sawala & Gould, 2017; Spaziani & Radicioni, 2020). Ultimately, sex-specific selection (potentially arising from sexual selection) likely drives these sex differences in aging hallmarks, and the triggers of sex differences in aging are closely coupled with sexual differentiation pathways. Males and females often differ in (i) genome composition (e.g., sex chromosomes); (ii) adult morphology and life-history (e.g., adult sexual size dimorphism, reproductive investment); and (iii) environments—both biological environments (e.g., hormonal milieu, microbiomes, parasites), and ecological

environments (e.g., partitioning of home ranges / breeding grounds, intra- and inter-sexual competition). How these biological and ecological differences between the sexes interact and impact the molecular drivers of aging and thus precipitate sex differences in aging is an important open question in the comparative biology of aging. Here, we summarize hypotheses and data related to genomic, morphological, and environmental differences between the sexes and how these relate to differences in senescence and lifespan. We end by suggesting future research emphases in this area.

2 | GENOMIC DIFFERENCES BETWEEN THE SEXES AND DIFFERENCES IN AGING

Genomic differences between the sexes can arise at fertilization. Thus, sex-specific aging may derive, in part, from differences in genotypes between males and females related to sex-determination mechanisms.

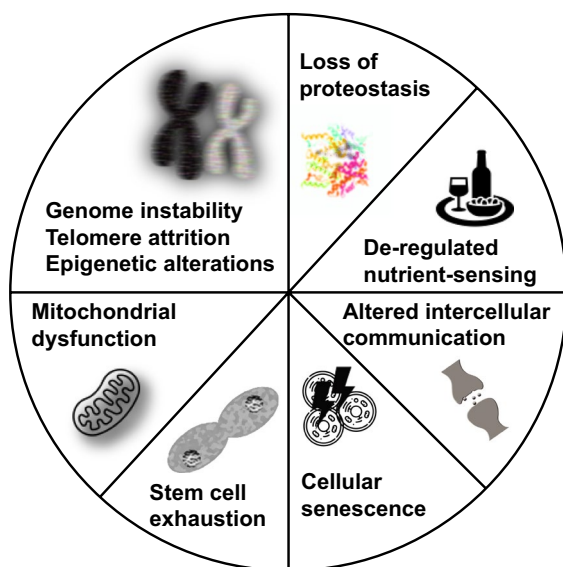
2.1 | Species with sex chromosomes

In species with sex chromosomes, the genomes of the two sexes differ in their sex chromosome complement. While other systems exist, most sex chromosomes generally fit into one of two categories: XX/XY systems, where males are the heterogametic sex (XY genotype) and females are homogametic (XX); and ZZ/ZW systems, where females are heterogametic (ZW) and males are homogametic (ZZ). There is considerable variation within these two sex chromosome types, as they have evolved multiple times independently (Bachtrog et al., 2014). Despite this variation, there is broad, empirical support for shorter lifespans in the heterogametic sex compared to the homogametic sex (Xirocostas et al., 2020), although with much variation (Marais et al., 2018). An analysis focused on tetrapods suggested that the sex-determination system (XY vs. ZW) explained $\frac{1}{4}$ to $\frac{1}{2}$ of the differences in the adult sex ratio (a proxy of sex differences in adult mortality) observed between species, depending on the heterogametic sex (Pipoly et al., 2015). A similar result was obtained in a study focused on amphibians (Cayuela et al., 2021). Data from the four core genotypes in mouse (animals with either XX or XY sex chromosome complement and either ovaries or testes generated by exploiting a translocation of the SRY gene to an autosome) shows that the presence of two X chromosomes improves lifespan, irrespective of the gonads (Davis et al., 2019). Together, the available data suggest that sex chromosomes might have a role in generating sex differences in aging (Marais et al., 2018).

Several potential explanations for the shorter lifespan of the heterogametic sex have been proposed. First, the “unguarded X” (or “Z”) hypothesis (Trivers, 1985) suggests that the heterogametic sex (i.e., with only one full sex chromosome X in males of XY species, and Z in females of ZW species) might express more deleterious morphological and physiological characteristics. This prediction derives from the observation that recessive deleterious X- or Z-linked alleles are likely



BOX 2 Molecular mechanisms of aging



Genomic instability: Increasing genome instability with age (i.e., rates of aneuploidy, somatic mutations, and dysregulation of transposable elements [TEs]) is well documented in many species. Evidence for a clear causal relationship to senescence is largely lacking, but data from model species suggest that improved DNA repair and increases in TE silencing can lead to longer lifespans. In addition, in humans, premature aging syndromes are linked to DNA repair and genome instability.

Telomere attrition: Telomere attrition in humans correlates with age, and telomere attrition is directly related to cellular senescence (a primary cause of inflammation) and altered gene expression in sub-telomeric regions (Dong et al., 2021). In mammals, the level of telomere maintenance with age depends on body size, with telomerase activity negatively correlated with body mass (Tian et al., 2018). Thus, there are a significant number of species documented, which lack telomere attrition, but nonetheless show physiological aging similar to species that show telomere attrition.

Epigenetic alterations: Epigenetic changes with aging are widespread and include changes to cytosine methylation (Wilkinson et al., 2021), histone modifications, and chromatin structure. Heterochromatin loss associated with aging occurs in several species and improved maintenance of heterochromatin extends health- and lifespan (Ngian et al., 2021; Wood et al., 2016). In addition, a recent study demonstrated that expressing the key reprogramming genes *Oct4*, *Sox2*, and *Klf4* in old mice resulted in the re-establishment of “young” cytosine methylation patterns and improved physiological functions (Lu et al., 2020), suggesting that epigenetic change underlies senescence.

Loss of proteostasis: To maintain cellular proteins in a functional state, protein production, folding, modification, and degradation have to be carefully balanced. This balance is lost with advancing age, leading to a variety of problems. A causal link to aging is supported by studies that report increased lifespan in animals overexpressing chaperone proteins that promote proteostasis (Bobkova et al., 2015; Hsu, Murphy & Kenyon, 2003; Morley & Morimoto, 2004; Morrow et al., 2004; Yokoyama et al., 2002).

Deregulated nutrient-sensing: Nutrient-sensing pathways in animals include the IIS (Insulin and Insulin-like growth factor [IGF-1] signaling), sirtuin, AMPK (AMP kinase), and mTOR (mechanistic target of Rapamycin) pathways. These pathways are responsible for assessing the body's nutritional needs and status, and their ability to do so decreases with age. Studies from several model species suggest that modulating these pathways genetically or by dietary restriction can impact life- and healthspan.

Mitochondrial dysfunction: Mitochondrial function tends to decline with age. At the same time, mtDNA mutations increase with age. The combined roles of mitochondrial function, oxidative stress, mutational load, and mitochondrial mass have been the subjects of decades of research. Notwithstanding, the precise mechanisms by which mitochondrial phenotypes contribute to senescence remain relatively unknown.

Cellular senescence: Loss of proliferative capacity is the main feature of cellular senescence (Di Micco et al., 2021). The accumulation of senescent cells leads to chronic localized inflammation. Senescent cells tend to have high levels of p16^{ink4a}, which inhibits cyclin-dependent kinases. Removal of cells with high p16 levels delays age-associated disorders in mice (Baker et al., 2011; Che et al., 2020). However, this removal also has negative consequences and does not always lead to the desired senescence-delaying effects (Grosse et al., 2020).

Stem cell exhaustion: Stem cell exhaustion refers to the shrinking pool of stem cells with age (Ren et al., 2017). Over time, stem cells lose their capacity to produce differentiating daughter cells while maintaining their stem cell properties. Currently, it is mostly unclear to which extent stem cell exhaustion contributes to aging in general.

Altered intercellular communication: With increased age, intercellular communications change and affect endocrine and neuronal communication between cells (see López-Otín et al., 2013) and references therein). Particularly impacted are immune functions, with inflammatory reactions tending to increase with age, while surveillance against pathogens and malignant cells decrease (Borgoni et al., 2021). The overall contribution, causal or correlative, of altered cell-to-cell communication (beyond inflammaging (Franceschi et al., 2018) is not clear (but see Yousefzadeh et al., 2021 for a promising transplant study).



to be masked by a dominant beneficial allele in the homogametic sex, but are exposed to selection in the heterogametic sex. Despite solid theoretical foundations, the unguarded X hypothesis has mixed empirical support (Brengdahl et al., 2018; Sultanova et al., 2018, 2020). Second, the “toxic Y” (or “W”) hypothesis (Brown et al., 2020; Marais et al., 2018; Nguyen & Bachtrog, 2020) suggests that the Y or W chromosomes (which are sex-limited) might account for sex differences in lifespan. Y-linked genetic variation for male lifespan exists in *D. melanogaster* (Griffin et al., 2015) and chinook salmon (McKinney et al., 2020). Consistent with the role of the Y chromosome in aging, repetitive DNA on the Y chromosome is de-repressed in older *Drosophila melanogaster* males, leading to mis-expression of Y chromosome repeats as a function of aging. Data from humans also support the presence of a toxic Y effect, as the presence of an additional Y chromosome in males (i.e., XYY) leads to a 10-year reduction in lifespan (Stochholm et al., 2010). In contrast, human males with an additional X chromosome (XXY, Klinefelter) only show a 2-year reduction in lifespan (Bojesen et al., 2004). Thus, either the “unguarded X” or “toxic Y” hypotheses may help explain the shorter lifespan of the heterogametic sex, but the relative importance in various species is unknown.

X (or Z) chromosome dosage compensation may also have sex-specific effects on lifespan—either by upregulation of the single sex chromosome in the heterogametic sex or by downregulation of the duplicate sex chromosome in the homogametic sex. Some taxa have evolved sex-specific regulation of the X or Z chromosome in the heterogametic sex, which is predicted to compensate for the haploid expression of X- or Z-linked genes (Ohno, 1967). In *Drosophila*, the X chromosome is upregulated in hemizygous XY males to equilibrate with the diploid dosage of the autosomes (Lucchesi & Kuroda, 2015). Misregulation of dosage compensation in males could explain shorter lifespan of *Drosophila* males. In comparison, in humans, where one copy of the X chromosome is inactivated in XX females (with some important exceptions, Tukiainen et al., 2017), biased (non-random) inactivation of one copy of the X over the other is associated with shorter lifespan (Chuaire-Noack et al., 2014; Gentilini et al., 2012; Ostan et al., 2016). However, as this process occurs in females, it does not explain the shorter male lifespan. Thus, while sex-specific gene regulation via dosage compensation might impact sex differences in aging in some species, we maintain that it is not a general mechanism underlying sex differences in aging across taxa because of the idiosyncratic nature of dosage compensation across eukaryotes (Gu & Walters, 2017; Mank, 2013).

2.2 | Species with haplodiploidy

Species where sex is determined by the presence/absence of chromosome pairs provide another opportunity to investigate the possible impacts of genomic differences between males and females on sex differences in aging. Species with haplodiploidy are best known among hymenopteran insects (e.g., bees, ants, wasps; female diploid, male haploid), but also include some scale insects and rotifers (Blackmon et al., 2017). In addition, some insects have sex differences in the elimination of the paternally inherited chromosomes, which

can result in effective haploidy of the somatic genome (Gardner & Ross, 2014). Social Hymenoptera are of particular interest in terms of sex differences in aging, as often the sexes differ significantly in terms of lifespan, but also because there often are multiple classes of females—extremely long-lived queens and sterile workers with lifespan more similar to the males. In these species, something similar to the “unguarded X” hypothesis might explain the sex differences in aging, as essentially the entire chromosome complement is unguarded in males, leading to the phenotypic manifestation of any deleterious alleles present (Smith & Shaw, 1980). However, this hypothesis does not explain the extensive lifespan differences between castes of one sex, such as the diploid sterile workers and the diploid fertile queens, suggesting that other aspects of these animals' biology (e.g., gene expression, environments, diet) precipitate these lifespan differences.

2.3 | The mother's curse: female-specific inheritance of mitochondrial genomes

An additional genetic difference between the sexes involves the inheritance of mitochondria, which typically are passed from mother to offspring. The matrilineal inheritance of mitochondria means that selection on mitochondrial genomes (mtDNA) occurs only in females, leading to the prediction that males may suffer a “mother's curse” from mtDNA alleles that are optimized for female-specific needs with respect to energy metabolism (Gemmell et al., 2004; Innocenti et al., 2011). Evidence for the mother's curse in animals is mixed (Dowling & Adrian, 2019), but it has been hypothesized to explain shorter lifespans in males than females (Camus et al., 2012; Frank & Hurst, 1996). For example, a study of Leber's hereditary optical neuropathy, a condition caused by mitochondrial mutations, discovered that these mutations lead to worse phenotypic outcomes in males than in females and are maintained in the population due to the matrilineal mtDNA transmission (Milot et al., 2017). The extent of the mother's curse may further depend on temperature for poikilotherms (Montooth et al., 2019), and we discuss how differences in temperature and energy metabolism can lead to sex differences in aging below. Moreover, the male-limited inheritance of the Y chromosome may result in male-specific adaptations that negate the mother's curse or even create a countervailing “father's curse” on autosomal loci (Ågren et al., 2019). The compounding effects of these sexually antagonistic selection pressures with sex-biased modes of inheritance could lead to sex differences in aging. Nevertheless, it is difficult to predict which sex should outlive the other without knowing the values of many different parameters in a variety of population genetics models. For this reason, the mother's and father's curses remain intriguing explanations for sex differences in aging, but are of limited predictive value.

3 | PHENOTYPIC DIFFERENCES BETWEEN THE SEXES AND DIFFERENCES IN AGING

In many species, in addition to the difference in sex organs, males and females also differ in a variety of features with varying degrees of



sexual dimorphism. Extreme sexual dimorphism is often associated with mating systems. When males physically compete for access to females, the males tend to be the larger sex and may develop weaponry such as horns or antlers (e.g., Bro-Jørgensen, 2007; Lindenfors et al., 2007; but note that in ~70% of bovids, females also have horns: Lundrigan, 1996). When females choose mates among males, male ornamentation in plumage, horns, vocalizations, or bright colors often occurs (Zuk & Simmons, 2018). In contrast, females may evolve ornaments in response to sexual selection or other selection pressures (Murray et al., 2018; Tobias et al., 2012). These ornaments can be costly to develop or maintain and thus may contribute to sex differences in aging (reviewed in Tidière et al., 2015). In addition, sperm competition might also be costly for males, thus impacting sex differences in aging (Lemaître et al., 2020). Sexual dimorphism also extends to a variety of morphological, physiological, and likely, molecular characteristics. In humans, for example, males and females differ in how their muscle develops and changes with age (Gheller et al., 2016). Experimental evolution studies on wheel-running in mice have resulted in sex-specific differences in lifespan, morphology, and physiology. For example, males are heavier than females in both high-running and control mice, but both sexes of the high-running strains are smaller than the control animals and the two sexes also show differences in body composition and corticosterone levels (Bronikowski et al., 2006; Castro et al., 2021). Given the wide-ranging phenotypes that show sexual dimorphism, they need to be considered as potential causes of sex differences in aging (Tobias et al., 2012).

3.1 | Sexual dimorphism in body size

Sexual size dimorphism is common in many groups. In mammals, males are often larger than females, whereas in reptiles (including birds) and insects, females are often larger than males. Ray-finned fish exhibit the widest range of sexual size dimorphism across animals, with variation from dwarf and parasitic males to males that are more than 12 times larger than females (Fairbairn, 2015). Factors influencing the extent and direction of sexual dimorphism in body size include competition for mates and resources, mating systems, predation risk, and diet. For example, selection for increased fecundity often favors large females (e.g., reptiles: Bronikowski & Arnold, 1999), while extensive competition among males for access to females favors large males (e.g., mammals: Andersson, 1994; Weckerly, 1998), and selection for a shorter time to sexual maturity can lead to smaller animals of both sexes.

Differences in selective pressures between females and males, and the resulting sexual dimorphisms in size, must be considered in the context of life-history strategies. For example, along the fast-to-slow pace-of-life continuum, suites of life-history traits undergo correlated evolution toward more fast-paced (fast growth, shorter lifespan) or slow-paced (slow growth, longer lifespan) (e.g., Gangloff et al., 2020; reviewed in Dammhahn et al., 2018). Selective pressures that cause the evolution of slower- or faster-paced life histories can differ between the sexes and result in significant variation in morphological dimorphism and lifespan (Fairbairn et al., 2007; Maklakov & Lummaa, 2013). The

brown antechinus (*A. stuartii*), a small marsupial, provides an extreme example, as all males die after mating, while ~10%–15% of females survive multiple mating seasons (Fisher & Blomberg, 2011). Phylogenetic constraints in sexual size dimorphism appear to be mostly absent; within many clades, sexual size dimorphism ranges from non-existent, to males being twice as large as females, or females being much larger than males (Bronikowski et al., 2011; Ceballos et al., 2013; Cheverud et al., 1985; Ota et al., 2010; Rohner et al., 2018). In at least some species groups (birds and mammals; Promislow, 1992; Promislow et al., 1992), sexual dimorphisms and sexual selection are linked to sex differences in aging (but see Lemaître et al., 2020; Tidière et al., 2015).

Sexual dimorphism in body size is relevant to sex differences in aging as there are several connections between body size and aging. Generally speaking, among species, larger animals live longer than smaller animals (Speakman, 2005), whereas within species, the opposite can be true [e.g., dwarf mice (Bartke & Brown-Borg, 2004; de Magalhães & Faragher, 2008) or dogs (Fleming et al., 2011; Kraus et al., 2013)]. This relationship between body size and lifespan is likely due to the fact that the pathways controlling lifespan and body size overlap at least partially. For example, dwarf mice are deficient for growth hormone, which leads to their small body size. However, the growth hormone deficiency also leads to increased insulin sensitivity and impacts insulin signaling (reviewed in List et al., 2021), which is an important modulator of lifespan. Interestingly, the lifespan extension seen in mice for growth hormone deficient animals is not seen in humans with growth hormone deficiencies (Bartke, 2021), suggesting that the link between growth regulation and lifespan is complex.

The allometric scaling of lifespan, that is, smaller animals living longer within species, however, is unlikely to explain sex differences in aging. For example, in primates, males tend to be larger than females, which leads to the prediction that males should live longer based on their body size, which is not normally seen. In addition, the environment, especially diet, also has significant impacts on body size and aging. Caloric restriction (CR)—within limits—often leads to increased lifespan, while high calorie diets lead to accelerated aging. Indeed, CR is known to extend lifespan in a sex-specific and strain-specific manner, which is well documented in mice and fruit flies (reviewed in Garratt, 2020 and Krittika & Yadav, 2019). In some insects, CR results in smaller, longer-lived animals. In the short-lived killifish *Nothobranchius furzeri*, CR extends lifespan of males, but not females (McKay et al., 2021). In *Caenorhabditis elegans*, CR increases lifespan in hermaphrodites, but does not impact the lifespan in males (Honjoh et al., 2017). These examples illustrate the complex nature of the body size/lifespan relationship and how sexual dimorphism in body size might contribute to sex differences in aging.

3.2 | Sexual dimorphisms related to differences in developmental timing

In addition, one needs to consider the potential impact of differences in growth patterns and developmental timing on sexual dimorphisms

that may result in sex differences in aging. While many species have genetically programmed growth cessation (i.e., determinate growth), species with indeterminate growth lack this limit and can theoretically continue to grow when resources are available and the environment permits. While male and female animals both begin development from a single identically sized cell, once developmental programs for each sex have been initiated, the two sexes have the potential to develop at different rates as they respond to different hormonal or environmental cues. This process can lead to large differences between females and males in age of maturity (Figure 3; de Magalhães et al., 2005), as well as sex differences in body size and other traits.

Sex differences in growth patterns are particularly common in poikilotherms (e.g., insects, fishes, and reptiles), and are of interest in aging biology because indeterminate growth can lead to indeterminate fecundity, which can change selection pressures in adult animals dramatically (Promislow & Bronikowski, 2006). In insects, the impact of growth rate and sex differences in developmental timing on body size is illustrated well by a study of black scavenger flies (Rohner et al., 2016), which focused on populations of *Sepsis neocynipsea* in North America (males larger than females) and Europe (females larger than males). While in most insects, females are the larger sex, Rohner and colleagues report growth rate differences between the sexes as well as a role for prolonged male development in the populations that show larger male body size.

In fish, Pacific salmon species (*Oncorhynchus* spp.) that are anadromous and semelparous exhibit varying degrees of sexual dimorphism and rapid senescence after spawning. Sexually mature sockeye salmon (*O. nerka*) are sexually dimorphic where males are generally larger, with deeper bodies and longer jaws than females (Johnson et al., 2006; Quinn, 2018; Quinn & Foote, 1994). Growth rates vary between anadromous salmon as they can spend 1–4 years feeding and growing to their final adult size in the ocean before returning to their spawning sites (Dittman & Quinn, 1996; Quinn, 2018), creating size-age variation at spawning and subsequent death. In contrast, in Atlantic salmon

(*Salmo salar*), 1%–6% of females survive after spawning, and return to the ocean where they can recover and spawn again (National Research Council et al., 2004; Tessier & Bernatchez, 2000). Thus, some Atlantic salmon are iteroparous, creating a unique population of older females.

In reptiles, plasticity in growth rates can be greater in one sex than the other (e.g., turtles: Ceballos et al., 2013, 2014; Ceballos & Valenzuela, 2011), reflecting sex-specific selective pressures that can impact life histories, including aging (e.g., Bronikowski et al., 2021; Hoekstra et al., 2018). These examples of sex-specific growth and development illustrate how sex-specific selection pressures can give rise to (or result from) these patterns. In turn, variation between the sexes in selection and generation time may give rise to variation in strategies of somatic maintenance between the sexes, and the molecular mechanisms that underlie such maintenance (see Box 2). While this variation between the sexes might impact sex differences in aging, it can be difficult to disentangle the effects of sex-specific growth rate, differences in absolute age at sexual maturity, and differences in body size. Likely, careful manipulations in model species will be needed, with the goal of altering one trait while keeping the others constant (for example, see Lind et al., 2017 for a study in *C. elegans*).

4 | ENVIRONMENTAL DIFFERENCES BETWEEN THE SEXES AND DIFFERENCES IN AGING

4.1 | Sex-specific responses to the environment

The environment experienced by the two sexes also needs to be considered as a potential factor impacting sex differences in aging. Due to genetic differences and sexual dimorphism in various phenotypes, males and females might experience and respond to the environment differently, which, in turn, can influence sex-specific trajectories of mortality and the molecular mechanisms underlying these trajectories. Temperature is a well-studied environmental variable with sex-specific responses. In general, at higher temperatures, animals have to expend more energy to maintain proteostasis as more chaperones, including heat shock proteins, are required to ensure proper protein folding (Somero, 2020). This gives rise to thermal critical maxima (and minima) that are species- and sex-specific (reviewed in Bodensteiner et al., 2021). These effects of heat on cellular and biochemical functions may explain why ectotherms live longer at colder temperatures (Conti, 2008; Keil et al., 2015; Miquel et al., 1976).

Heat stress differentially impacts males and females in several species. In *Drosophila melanogaster*, male fertility is impacted more significantly than female fertility at higher temperatures (Zwoinska et al., 2020). In reptiles, differential effects of temperature on growth and immune function in the two sexes have been reported in common garden experiments (e.g., Palacios et al., 2020), including effects on survival (Addis et al., 2017). Addis and colleagues report for garter snakes that the impact of temperature depends on sex as well as

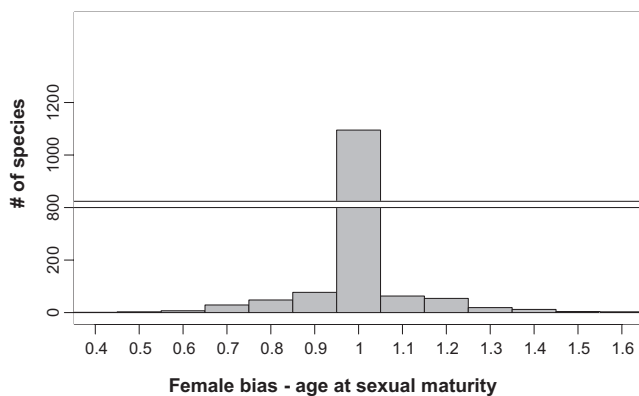


FIGURE 3 Variability in female age bias at sexual maturity across chordates. Female age bias is defined as female maturation age divided by the mean maturation age of both sexes. This distribution is centered at 1 (i.e., no age bias), with range from 0.42 to 1.62, with equal tails. Data from AnAge (birds contribute 48% of the data, mammals 40%)



ecotypes, with one ecotype (lower elevation, fast growth) showing increased female mortality at low temperatures, which is absent in males and both sexes of the second ecotype (higher elevation, slow growth; Addis et al., 2017). Similarly, male fertility is negatively impacted by high temperatures in many mammals, often with a lesser effect seen in females (Takahashi, 2012). Given the importance of energy allocation toward reproduction versus somatic maintenance for the progression of aging (Kirkwood, 1977), these examples illustrate how temperature might impact sex differences in aging.

Studies in seabirds, snakes, and beetles further illustrate how other environmental effects can influence sex differences in aging. A study from a seabird, the imperial shag *Leucocarbo atriceps*, revealed a complex interaction between fledgling weight, resource availability, and social environment (sibling number) (Svagelj et al., 2021). Males are typically larger than females, and in poor years, chicks of both sexes without siblings showed worse performance. In good years, male chicks weighed less in the presence of a sibling, while female fledgling weight was unaffected by the social environment (Svagelj et al., 2021). In great skua (*Stercorarius skua*), females are larger than males, and female chicks typically grow faster than males, but poor environmental conditions led to slower growth in female chicks than in male chicks (Kalmbach et al., 2009). Sex-specific effects of development under poor nutrition also have been studied in garter snakes (Holden et al. 2019) where females, but not males, had significantly lower adult survival when they developed under poor nutrition (despite exhibiting catch-up growth when switched to a high-nutrient diet pre-maturation). In humans, intra-uterine growth restriction leads to negative outcomes more often in males (reviewed in Cheong et al., 2016). These findings demonstrate that identical environments can have very different impacts on the two sexes, leading to suboptimal outcomes in one sex, but not the other, potentially impacting survival and aging.

A sex-specific environmental effect on lifespan has been explicitly demonstrated, for example, in the seed beetle *Callosobruchus maculatus* (Sanghvi et al., 2021). Sanghvi and colleagues manipulated larval density to determine the impact of early life environment quality on flight performance, fecundity, and lifespan. They found that female fecundity and lifespan are negatively affected by poor larval environment, while male fecundity and lifespan are not affected (Sanghvi et al., 2021). Another example is a study of Seychelles warbler, *Acrocephalus sechellensis*, which demonstrates that the presence of non-breeding or co-breeding helper females increased survival for older dominant females significantly, but did not do so for dominant males. Interestingly, dominant females lacking helpers showed increased rates of telomere attrition compared to females with helpers, while male telomere attrition rate was not impacted by the presence of helpers (Hammers et al., 2019). These two studies demonstrate that identical environments can have very different impacts on survival and aging for males and females and hint at possible mechanisms. However, assigning causal relationships in these kinds of studies can be difficult as even simple manipulations can have a variety of effects and impact various molecular pathways linked to aging.

Environmental factors also contribute to sequential hermaphroditism in fishes, where there are three patterns of sex change: (1) protogynous (female-to-male), (2) protandrous (male-to-female), and (3) serial bidirectional (Avisé & Mank, 2009; Edgcombe et al., 2021). All patterns of functional sex reversal include restructuring of the gonad plus changes in behavior and morphology, which can include body size (Godwin, 2009; Nakamura et al., 2015; Warner, 1984). These data show that in addition to morphology and behavior, biological sex can be plastic. How this impacts age-specific trajectories of mortality and lifespan is unknown. A dominant theory explaining the adaptive significance of the timing, direction, or pattern of sex change is the size advantage model, which details how sex change is adaptive when the reproductive value is greater when one sex is small and the other sex is older and thus larger (Ghiselin, 1969; Kazancıoğlu & Alonzo, 2010; Munday et al., 2006; Warner, 1975). The timing of sex change should therefore maximize expected lifetime reproductive success (Warner, 1988). Sex-specific size advantages associated with different mating systems will drive the direction of hermaphroditism within a species (Munday et al., 2006), and thus environmental factors affecting size attainment can directly influence sex change and alter individual lifespan. These sex-specific responses to the environment potentially impact aging.

4.2 | Sex-specific environments

In addition to sex-specific responses to shared environments, sex-specific aging can occur if the environments experienced by the two sexes differ, which can begin in utero in mammals and viviparous reptiles, or at oviposition if mothers alter egg contents in an offspring-sex-dependent manner. Similarly, differences in preferences between the sexes for habitat, thermal profiles, and diet can also result in sex-specific environments. Such sex-specific environments are the norm for many species, often due to sex-dependent dispersal behavior. Many mammals form matrilineal social groups with males dispersing among groups, which results in males and females experiencing very different social environments as sub-adults (e.g., primates: Bronikowski et al., 2011). Indeed, such male dispersal is seen in most polygynous mammal species (Clutton-Brock & Lukas, 2012). As another example, most temperate bat species form female-only "maternity colonies" with males living elsewhere. Even though both sexes are migratory, only females return to these maternity colonies (Senior et al., 2005). While female-biased dispersal was thought to be typical of socially monogamous birds (Greenwood, 1980), phylogenetic analysis has not confirmed an association between mating system and sex-biased dispersal (Mabry et al., 2013).

In addition to sex-specific environments due to dispersal behavior, competition environments may also differ between the sexes. In many polygynous mammals, such as elephant seals, males aggressively compete with other males for access to mates while females avoid such dangerous conflicts and typically spend time obtaining sufficient resources for rearing offspring (reviewed in Fairbairn,



2015). Such competition among males can be energetically costly and cause injury and death. Mathematical modeling suggests that this sex-specific “live fast, die young” strategy can evolve under conditions with short mating seasons and intense competition among individuals of one sex, which results in increased investment into reproductive effort coupled with minimal investment into post-mating somatic maintenance (Fisher et al., 2013). While this type of reproductive strategy is rare in mammals, it is widespread in other taxa, such as many fishes, where differences in the competitive environments experienced by males and females lead to different life-history strategies and sex differences in lifespan and aging. It also should be noted that these sex-specific life-history strategies do not only result in sex-specific environments, but are linked also to the evolution of size dimorphisms and other traits that might impact aging independently.

Interactions between the two sexes and parental care represent other aspects of a sex-specific environment with the potential to impact sex differences in aging (Klug et al., 2013). In many species, mating itself (irrespective of successful fertilization) can impact lifespan. In *Drosophila melanogaster*, male lifespan is reduced by mating and by even just the perception of the opposite sex (Gendron et al., 2014). Likewise, females living in the presence of males have shorter lifespan than expected when accounting for egg production (Harvanek et al., 2017; Landis et al., 2021; Partridge & Farquhar, 1981; Partridge et al., 1987), but a recent study found surprisingly small effects of mating on lifespan across 15 *Drosophila* strains (Hoffman et al., 2021). In many species, parental care is strongly dimorphic (reviewed in Clutton-Brock & Scott, 1991). For example, in many mammals, females will provide for their offspring, first in utero, later nursing their young, and eventually training them to provide for themselves. The contribution from the male parent ranges from sperm-only to the extended participation in child rearing by both parents seen in humans. Sexually dimorphic parental care is also seen in archosaurs (birds and crocodilians) and cichlid fishes (reviewed in Gans, 1996; Reynolds et al., 2002). Although most reptiles do not show extensive parental care per se, females and not males often engage in nesting behaviors (such as digging nests in oviparous species, and cessation of foraging in viviparous species: reviewed in Gans, 1996; Reynolds et al., 2002). A study of 37 Western Palearctic bird species found parental care to significantly impact sex differences in lifespan, while size dimorphisms did not (Owens & Bennett, 1994). Given the potential for sex differences in energy investment in reproduction, offspring survival, and parental care, this energetic dimorphism can lead to a sex difference in somatic maintenance and, ultimately, senescence and lifespan.

4.3 | Species experiencing a range of environments

The previous two sections illustrate that both physical (e.g., temperature, resource availability) and behavioral (reproduction, competition) environments can impact sex differences in aging and lifespan.

However, there are interesting intraspecific polymorphisms that provide evidence that sex differences in aging are robust across diverse physical environments (e.g., temperature, altitude, seasonality) and behavioral variation, although the magnitude of these sex differences can change. Some marine turtles, for example, have wide ranges that span from North America to Australia. Interestingly, similar sex-specific aging trajectories in loggerhead turtles occur in populations that differ substantially in their ages of sexual maturity (20 years in N. America vs. 35 years in Australia; Mayne et al., 2020). In contrast, one population of painted turtles along the Mississippi River exhibits sex-specific lifespans and aging rates, whereas other populations do not (Congdon et al., 2003; Reinke et al., 2020). Additionally, garter snakes with populations of fast- or slow-aging snakes (at low and high altitude, respectively) show greater skew in male vs. female reproductive success in the fast-aging populations and exhibit sex-specific effects of a SNP in mitochondrial Cytochrome B on metabolic rate and aging/lifespan (Gangloff et al., 2020). Mexican cave fish (*Astyanax mexicanus*) are a particularly fascinating example of a species with a sex-by-habitat interaction in the evolution of metabolic traits (Riddle et al., 2021), but without sex-specific or habitat-specific lifespan (Riddle et al., 2018). Migratory species are also of interest, as there can be migratory and non-migratory populations that experience vastly different environments and stresses, but still show similar levels of sex differences in aging and lifespan. A recent comparative study of birds and mammals showed that migrant species and populations tended to have a faster-paced life-history strategy relative to non-migratory species (Soriano-Redondo et al., 2020). A study of Chesapeake Bay striped bass demonstrated that migrations tended to be undertaken only once animals achieved a specific size/age and that the non-migratory animals experienced higher mortality than migratory animals. Interestingly, the sex distribution of migratory animals was skewed toward females, but it is unclear if this is due to differential mortality or aging between the sexes or sexual dimorphism in body size (Secor et al., 2020). These examples illustrate how studying species experiencing diverse environments can provide insights into sex differences in aging.

Finally, short-lived species where subsequent generations experience different environmental conditions are informative. Insects that have spring versus winter generations might have different phenotypic morphs, but still experience sex differences in aging and lifespan. For example, *Drosophila suzukii* overwinter as adults, and females will lay eggs after the cold period, and these winter morphs show significantly longer lifespans than summer morphs (Shearer et al., 2016). In the desert locust, the gregarious morph has a shorter lifespan than the solitary morph. In monarch butterflies, summer-time “reproductive” individuals showed far more pronounced sex differences in aging than autumnal migratory individuals in reproductive diapause (Herman & Tatar, 2001). These examples illustrate that, for some species, intrinsic factors might be more important than extrinsic environmental factors in generating sex differences in aging, but that for other species, the interaction between intrinsic (biological) and extrinsic (environmental) factors might be key.



5 | OPPORTUNITIES FOR RESEARCH INTO SEX DIFFERENCES IN AGING

As the examples in the previous sections illustrate, there is immense variation among male and female animals in chromosome complement, morphology, and life history, as well as in the contribution of environment to sex differences in aging. In this section, we focus on genomic differences between the sexes as an especially promising avenue to understand sex differences in longevity and aging. Our premise is that careful selection of species can reveal how genome content and dynamics can contribute to sex-specific aging in both inter- and intra-species experimental designs. Below, we highlight three particularly promising areas related to genome differences between males and females: sex chromosomes, genome instability, and gene regulatory cascades.

5.1 | Sex chromosomes in sex-specific aging

As highlighted in Section 2, genomic differences between the sexes can arise at fertilization due to sex chromosomes. Sex differences in aging that may arise from sex chromosomes are likely due to the differences in gene content between the X and Y (or Z and W). In some animal groups, including mammals, *Drosophila*, and caenorhabditid worms, the X and Y (or Z and W) are highly differentiated, or heteromorphic. The Y or W has fewer functional genes and more repetitive elements compared to the X (or Z) (Charlesworth & Charlesworth, 2000; Graves & Marshall Graves, 2006; Rice, 1996). However, sex chromosome differentiation occurs across a continuum, and numerous animal species lack heteromorphic sex chromosomes having instead poorly differentiated, or homomorphic, sex chromosomes, which still retain many of their functional genes and lack significant increase in repetitive elements.

Two competing hypotheses for sex-specific aging are the unguarded X/Z (more genes on the larger X/Z chromosome reveal unmasked deleterious alleles in the heterogametic sex), and the toxic Y/W (more repetitive sequence on the Y/W are deleterious in the heterogametic sex) (see Section 2). In the first case, comparison across species with an X (or Z) with more genes, for example, on a larger chromosome, would predict a greater longevity differential between males and females due to the number of genes impacted by hemizygoty. We might also predict a similar effect in haplodiploid species where the entire genome is effectively unguarded in the haploid sex, with all deleterious alleles being unmasked. The greatest challenge with haplodiploid species is that sexual dimorphisms in life-history strategies often overwhelm lifespan differences, but solitary Hymenoptera could be promising models that overcome this challenge. Under the unguarded X/Z hypothesis, we would also predict little to no sex differences in aging in species that lack heteromorphic sex chromosomes (e.g., homomorphic or lack of sex chromosomes altogether, such as species with environmental sex determination). Comparisons among closely related taxa with and without heteromorphic sex chromosomes would resolve this question. Alternatively, under the toxic Y/W hypothesis, comparison across species with a Y or W chromosome ranging in size from smaller to larger predicts a greater sex differential

in aging in species with larger sex-specific chromosomes (i.e., Y or W). Similarly, we predict no observable effect in XX/XO or ZO/ZZ species where the Y or W has been lost entirely (Cochran & Ross, 1967; Fraïsse et al., 2017; Voelker & Kojima, 1971). Data from two *Drosophila* species illustrate this approach. Comparing *D. pseudoobscura* and *D. miranda*, both species with XY sex determination, but with Y chromosomes of different evolutionary age and size (*D. p.*- old, smaller; *D. m.*- young, larger), *D. miranda* shows more expression and transpositions of deleterious Y-linked transposable elements (Wei et al., 2020), illustrating how a size-dependent toxic Y effect might operate.

One limitation of inter-species comparisons is that there are confounding differences among species other than sex chromosomes that may also affect aging. Thus, a complementary approach to comparing different species is to examine the effect of Y-linked variation in aging within species that have documented Y polymorphisms (i.e., SNPs, copy number variation in repeats, structural variants). For example, Y chromosome haplotypes in *D. melanogaster* have *trans* effects on gene expression and chromatin throughout the genome in both XY males and XXY females (Lemos et al., 2008, 2010). Females do not transcribe any Y-linked genes, and therefore, the effect of the Y chromosome on female transcriptional regulation is most likely caused by the chromatin content of the Y chromosome. Comparing aging in males and females carrying different Y chromosome types would allow a direct test of the effect of different Y chromosomes. Other *Drosophila* species have documented Y chromosome polymorphisms (Archer et al., 2017; Branco et al., 2013; Dobzhansky, 1937), which offers the exciting possibility of performing both within and among species comparisons in this model genus.

5.2 | Genome instability in sex differences in aging

Following from the above hypotheses on the direct role of sex chromosomes on sex-specific aging, there may exist genome-wide phenomena that indirectly derive from sex determination. Animal species range in genome differentiation from heteromorphic sex chromosomes to species with haplodiploidy, where females have twice the genomic content of males, to environmental sex determination, where genome content is identical between the sexes. Of particular interest is closely related species that show different levels of genome differentiation or different modes of sex determination. In these species, it might be possible to untangle the impact of genomic differences on sex differences in aging from other factors such as life-history strategies or environmental factors. Such species groups include both vertebrates—reptiles (turtles, squamates), fish [Neotropical silversides (*Menidia* spp.), Poeciliids]—and invertebrates (marine worms, parasitic nematodes) (Janzen & Paukstis, 1991; Janzen & Phillips, 2006; Sabath et al., 2016; Tree of Sex Consortium, 2014). For example, turtles have evolved several modes of sex determination including XX/XY and ZZ/ZW sex chromosomes and temperature-dependent sex determination (TSD) (Bista & Valenzuela, 2020). Indeed, turtles and



lizards with sex chromosomes tend to live shorter lives than their TSD counterparts (Sabath et al., 2016). Yet, evidence of turtle senescence is mixed (reviewed in Hoekstra et al., 2020), and the sex specificity of demographic senescence is largely unstudied in turtles (Bronikowski et al., 2021). Whether measures of genome stability (such as DNA repair efficiency, chromosome accessibility, TE dysregulation, epigenetic modification) change in an age-specific, sex-specific, or age-by-sex-specific manner in closely related species with variable sex-determining mechanisms are unknown, yet could provide insights that would help disentangle sex determination from sex-specific aging.

Sex chromosomes can be lost or newly evolved, even if the sex-determination system is constant (Furman et al., 2020). Sometimes, sex chromosomes are differentiated, leading to increased transposable element load or heterochromatin levels in one sex but not the other, while in closely related species, the sex chromosomes might show minimal levels of differentiation, as seen in related poeciliid fishes (Darolti et al., 2019). Sex chromosomes also differ in size, and, in some species, the sex chromosomes can lead to significant differences in overall genome size between males and females [e.g., in *Drosophila virilis* females have the larger genome, while in *D. persimilis*, males have a larger genome (Hjelman et al., 2019)]. Among species with old sex chromosomes, such as mammals, much of this variation is caused by expansion and rearrangement of ampliconic regions (Brashear et al. 2018; Hughes et al. 2010). Thus, beyond the direct effect of sex chromosomes, genome size, transposable element content, and heterochromatin fraction (and concomitant gene expression) are genomic features that vary between sexes and species and have the potential to impact sex differences in aging by their impact on genome stability. Here again, comparison within and among species of *Drosophila* or fishes with neo-sex chromosomes, such as sticklebacks (Ross et al., 2009) or African cichlids (Gammerdinger & Kocher, 2018), would allow for the separation of sex-specific aging from sex chromosomes related to genome stability.

Finally, it is possible to select species to investigate the impact of dosage compensation systems and other sex chromosome regulatory pathways on sex differences in aging. In addition to the genome content, how specific chromosomes, in particular the sex chromosomes, are regulated in males and females can differ significantly. Best known are the dosage compensation systems. These systems, like the dosage compensation complex that upregulates X chromosome genes in *Drosophila melanogaster* males, impact one sex, but not the other. If these sex-specific regulatory pathways are costly or are likely to break down with age, they might contribute to sex differences in aging. The recent report of environmentally sensitive dosage compensation in turtles with ZZ/ZW sex chromosomes, which is also age- and tissue-dependent (Bista et al. 2021), adds to the complexity of factors potentially affecting sex-specific aging. Selecting species with similar genomic features that differ in sex chromosome regulation might provide insights into how these pathways impact genome instability and/or sex differences in aging. These comparative studies can then be complemented by work in

model species, where either the dosage compensation system or genomic characteristics can be manipulated to test specific hypotheses. Comparative studies across diverse taxa in conjunction with directed experiments in model organisms have the potential to lead to new insights into how dosage compensation systems might impact aging.

5.3 | Identifying regulatory cascades that control sex differences in aging

Another area of research that we believe could benefit from expanded use of comparative studies concerns the gene regulatory cascades that contribute to aging [e.g., Insulin-like Signaling (IIS, p53)] and sex differences in aging (McGaugh et al., 2015; Passow et al., 2019; Tower, 2017). Gene regulatory cascades are often pleiotropic, and currently, it is unknown if the interaction between gene regulatory cascades and sexually dimorphic genomes might contribute to sex differences in aging. For example, the IIS pathway, implicated across animal diversity in regulating aging through nutrient-sensing and stress responses, interacts with sex-determination mechanisms (Graze et al., 2018) and alters sexually dimorphic gene expression. As well, signaling through the IIS network can differ between the sexes and the sexes can differ in their responses to treatments that alter IIS signaling (reviewed in Towers, 2017). Even in non-traditional species, sex differences in IIS gene expression and protein levels have been observed (e.g., Crain et al., 1995; Reding et al., 2016). For example, master sex-determination genes in vertebrates frequently encode proteins in the TGF- β (Transforming growth factor beta) signaling pathway (Pan et al., 2021), which regulates many cellular processes (Derynck & Budi, 2019). TGF- β interacts with the highly evolutionarily conserved IIS/mTor signaling network (e.g., Narasimhan et al., 2011), which underlies trade-offs between reproduction and survival. It is possible for the sex-determination pathway to have sex-specific pleiotropic effects on aging. Another gene set of interest are imprinted genes, as they often impact growth patterns in a sex-specific manner (Patten et al., 2014). Comparative transcriptome studies and co-expression networks across species with diverse sex-determination systems would help to distinguish between pathways that impact aging in both versus just one of the sexes. These studies would also reveal the difference between species- or clade-specific mechanisms and mechanisms that act globally across animal lineages. To reach this level of understanding will likely require the collaboration of scientists from a variety of disciplines, as deep, omics-level data sets will be needed in addition to a comprehensive understanding of the organisms, their physiology, and life history.

One important feature to consider when investigating regulatory pathways relevant to sex differences in aging is the question of how sexual differentiation is accomplished, that is, via a cell autonomous system or via a hormonal system that affects cells across the body (Bachtrog et al., 2014). In cell autonomous systems



(such as seen in birds, *Drosophila* and nematodes), individual cells provide the genetic information to determine their sex, although signals from other cells might have some impact (Murray et al., 2010). In contrast, in cell non-autonomous, hormonal systems (i.e., in mammals, but see Arnold et al., 2013), specialized cells in the gonad produce sex-specific hormonal signals that are perceived by the rest of the body and result in a response to this signal, either male or female differentiation. While in species with hormonal sexual differentiation all cells are of the same genotypic and phenotypic sex (precluding somatic mutations), in species with cell autonomous sex determination an individual can be a mosaic of cells with different sexual phenotypes, male, female, or neither. This potential for mosaicism in species with cell autonomous sexual differentiation is seen in gynandromorphs, individuals where a portion of the body shows female characteristics while the rest shows male characteristics. Gynandromorphs have been reported for butterflies, insects, birds, and rodents, which is unexpected given their hormonal sex-determination system (Major & Smith, 2016; Nakamura, 2009). Gynandromorphs have the potential to provide insights into the complex interactions between cell autonomous and non-autonomous regulators of sex differences that can occur (e.g., see data from the four core genotypes model in mouse; Arnold & Chen, 2009). Understanding how cells perceive sex and what their identity is will be critical to correctly identifying regulatory pathways that contribute to sex differences in aging.

5.4 | Taxon selection for comparative studies of sex differences in aging

Species selection will be critical for comparative studies of sex differences in aging. As the examples in earlier paragraphs illustrate, there are many taxa that can provide valuable insights into sex differences in aging (Figure 4). In our opinion, the most promising taxa are those species where most of the factors that are likely to influence aging and sex differences in aging are constant, while ideally only one factor of interest is variable. For example, if our goal is to investigate the role of sex chromosomes, we would choose closely related species from a group, such as the geckos or turtles, where sex chromosomes are present in some species and absent in others. In contrast, if our goal is to investigate the role of size dimorphisms, we would choose species from the same species group, with similar life histories and environments, with one set of species showing sexual size dimorphism, and the other lacking it, with "replication" being provided by multiple independent evolutionary events. Similarly, if we are interested in understanding if warmer climates exacerbate sex differences in aging, we could choose species with wide geographic ranges and compare populations from different parts of the temperature cline. Species that reverse typically seen patterns are also of great interest, such as bird species where the female is larger or more colorful than the male (Amundsen, 2000; Edward & Chapman, 2011). Given the immense variation in both sex differences in aging, as well as in features of interest such as

sex-determination mechanisms, genome size, phenotypic dimorphisms, and more, it is possible to find suitable species groups to investigate a range of hypotheses and include "natural replication" across different taxonomic groups.

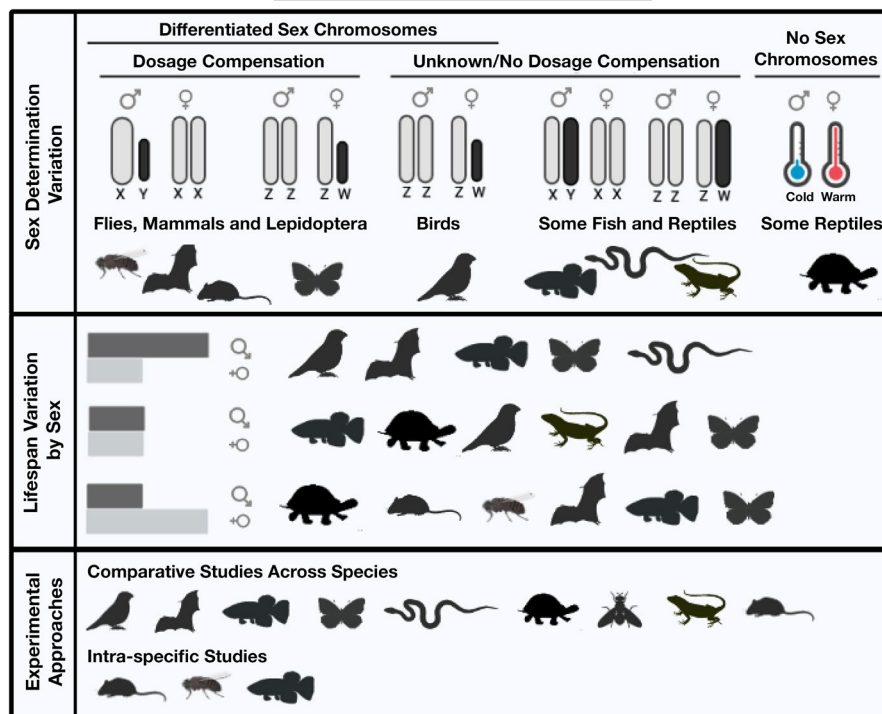
Species that exhibit sequential hermaphroditism (individual begins life as one sex, changing to the other sex sometime later in life), are documented in at least 27 families spread across nine teleost orders (Awise & Mank, 2009). Several species of African reed frogs (Grafe & Linsenmair, 1989) would be of great interest, as they could reveal if and how aging trajectories change with a sex change. Ideally, in each case, we would identify two or more species or populations of interest from more than one major branch of the animal tree of life to ensure that what we observe is a general phenomenon rather than a species-specific oddity. Likely, this approach will require biologists working with model species, lab-tractable species, captive populations, and wild populations. Despite the inherent challenges in this approach, strategic utilization of the immense variability in both sex differences in aging, as well as the factors hypothesized to control them, is possible and has great potential for the study of sex differences in aging.

5.5 | Study designs and data types for comparative studies of sex differences in aging

In experimental designs for testing hypotheses of sex-specific aging, both sexes at various adult ages are needed. However, it can be difficult to define comparable cohorts and samples in diverse animal taxa. Research communities focused on particular taxa typically have an agreed-upon standard for adults, but these standards often do not translate easily among species groups. For example, many research communities report age as time after sexual maturity, but insect researchers typically report age as time after eclosion. As well, the age of sexual maturity can be different between males and females, which begs the question of whether absolute age versus elapsed age since maturity is the appropriate chronological variable. Similarly, the developmental time prior to hatching can differ between the sexes. Based on our discussions with biologists working with a range of species, there is no simple solution to this problem. However, reporting age according to the species standard (age from eclosion or sexual maturity, etc.), absolute age, and age as a percentage of the maximum lifespan is an approach that allows researchers to compare across species as distinct as insects, fishes, and mammals. For example, defining "young adults" as the first quartile of adult lifespan and "old adults" as the fourth quartile of adult lifespan allows for comparable data sets to be collected from a variety of species (see Ronget & Gaillard, 2020 for additional ideas).

Which tissues to sample are another important question to solve if diverse species are included in a comparative study of sex differences in aging. With studies that span, for example, insects, fishes, and mammals, the task to identify comparable tissues becomes difficult. This issue is further complicated when wild populations are sampled, as the types of tissues that can ethically and efficiently be sampled in a

FIGURE 4 Example taxonomic groups for comparative studies of aging. Species with diverse sex determining mechanisms include those with heterogametic sex chromosomes, non-differentiated sex chromosomes, and environmental sex determination (warm temperature-dependent female determination highlighted here; various forms of TSD are found in many reptiles). Species with contrasting patterns of sex-specific lifespan include species with male-biased, female-biased, and unbiased lifespan. And species with inter- and intra-specific variation in aging include diverse wild population and laboratory model species



field setting are very limited. Given that data from humans and mice indicate that tissues may have tissue-specific aging rates (e.g., as in cytosine methylation; Bell et al., 2019; Kling et al., 2020; Zupkovitz et al., 2021), deliberate tissue choice is important. Likely, no “one size fits all” solution is possible, but tissues of interest include muscle, brain, and germ line, as these tissues show clear impacts of senescence in most species. Experimentalists will have to carefully review their choices for which tissues can realistically be sampled and carefully consider their choice in light of the overall study goal.

Finally, the analyses to be conducted on sampled tissues will have to be chosen. Again, data type will depend on the ultimate study goal, but a common minimal set of data and meta-data might be collected from a large number of species to allow for integration between studies. Meta-data should include demographic information about the individual sampled (age, sex, tissue, growth conditions, or location for wild species). Age might be difficult to determine, in particular in wild populations, which might need to be considered during the study design. Cytosine methylation clocks provide some promise for determining the age of wild animals that are not part of tagged or monitored populations (for example, see Robeck et al., 2021; Wilkinson et al., 2021). Additional data collected on individuals sampled will depend on the specific question under study, but information about size dimorphisms and overall physical health would be helpful. Finally, molecular measures of aging that can be collected include nuclear and mitochondrial DNA sequences to measure somatic mutations and DNA damage, transcriptomic data to measure aberrant gene expression and activation of transposable elements, telomere length to identify signs of telomere shortening, and chromatin integrity (Figure 5). Additional measures could be DNA damage repair efficiency, levels of stress hormones or antioxidants, the proteome, or metabolites. Limitations on what types of data can be

collected will likely depend on the circumstances of collection (field versus laboratory setting), as well as the amount of tissue that can be collected. For example, while 50 mg of tissue—sufficient for transcriptome analysis by RNA-seq and chromatin integrity analysis by ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing)—can easily be collected from a larger animal, for small animals such as many insects, this tissue amount requires the dissection and pooling of dozens of animals. Similarly, in a laboratory setting samples can be flash frozen immediately to preserve them for metabolomic or proteomic analysis, but in a field setting, tissue preservation methods are often much more limited, and may not be compatible with proteomic or metabolomic analyses. DNA-based assays are typically compatible with field-collected samples, but some chromatin and transcriptome assays are possible as well. Given continued development of methods that work with smaller and smaller samples, a promising strategy might be to focus on DNA-based assays in the short term and to store additional available samples for future investigation of other molecular aspects of aging.

6 | OUTLOOK

Comparative studies using both intra- and inter-specific experimental designs across the animal kingdom represent a promising opportunity to gain new insights into the origins of sex differences in aging. Technological advances in next-generation sequencing among other methods have made assays that were until fairly recently restricted to model and laboratory species adaptable to virtually any species of interest. In addition, the amount of tissue needed for these assays has decreased significantly, making it now possible to apply a variety of omics approaches in species across the tree of life. Other assays also

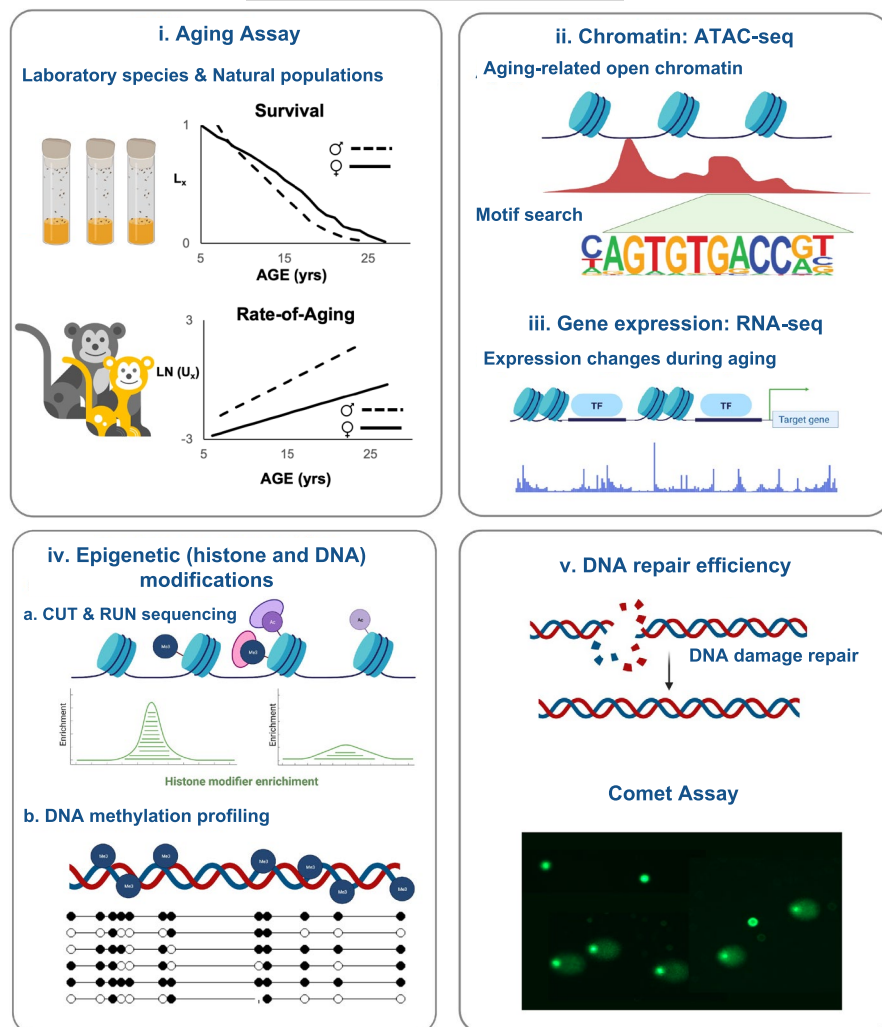


FIGURE 5 Example study design focused on genome stability changes with advancing age. i) First select a species that has sex-specific variation in demographic rate-of-aging (e.g., females live longer and age slower than males as pictured here for yellow baboons, Bronikowski et al. 2016). ii) Measure age-related accessible (eu)chromatin and iii) concomitant gene expression. iv) Measure additional age-related features of the epigenome. v) include functional assays of genome stability such as DNA repair efficiency. These genomic, epigenomic, and functional data can be integrated in deep learning pipelines to develop multi-variate indices of sex-and-age specific change

have become more sensitive, and non-invasive alternatives are becoming more available, meaning that more senescence-related characteristics can now be measured in more species than ever before. The most promising comparative studies will involve biologists from a variety of subdisciplines working together to tackle the question of sex differences in aging from different angles and with complementary approaches. Support from funding agencies and university administrations for these highly interdisciplinary studies will be needed, but the high potential for impact makes these studies worth pursuing.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

NCR: Conceptualization and Supervision/Project administration. AMB, RPM, PRB, JRW, JEM, EL, GSW, NV, and NCR: Writing—original draft preparation. AMB, RPM, PRB, JRW, JEM, EL, GSW, NV, NCR, AMC, JpDm, J(E)D, AEE, TG, RMG, KEG, JAK: Writing—review and editing. NCR, PRB, J(E)D, JEM: Visualization. NCR, JRW, EL: Funding acquisition.

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