Molecular Signatures of Aging in the Hippocampus of Rhesus Macaques

by

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A dissertation accepted and approved in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in Anthropology

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Spring 2026

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DISSERTATION ABSTRACT

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Doctor of Philosophy in Anthropology

Title: Molecular Signatures of Aging in the Hippocampus of Rhesus Macaques

Understanding why humans are uniquely susceptible to neurodegenerative diseases (e.g., Alzheimer's disease) requires insight into how the molecular architecture of the aging brain is organized and regulated – and how that organization may differ across primates with different levels of vulnerability to neurodegenerative diseases. This dissertation investigates molecular signatures of aging in the hippocampus of rhesus macaques (*Macaca mulatta*), a long-lived nonhuman primate that shares key features of brain organization, life history, physiology and genetic similarity with humans, making it a powerful translational model for studying primate brain aging. Using a multi-omic approach integrating bulk RNA-sequencing, reduced representation bisulfite sequencing, and small RNA-sequencing from matched hippocampal tissue samples spanning the full adult lifespan (~3-35 years of age), I characterize how gene expression, DNA methylation, and microRNA expression differ and interact across the lifespan in a brain region central to learning, memory, and neurodegenerative vulnerability.

Across all three molecular layers, I find evidence for several hallmarks of aging, including epigenetic alterations, inflammatory signaling, disrupted intercellular communication, and pathways associated with mitochondrial dysfunction. Critically, molecular aging in the macaque hippocampus does not always follow strictly linear trajectories. In fact, all three omic datasets reveal nonlinear molecular trajectories with a recurring midlife transition centered around ~10 years of age in macaques – a period that corresponds roughly to ~30 years of age in humans. This convergence across independent molecular datasets suggests that midlife may represent a critical

window of regulatory reorganization in the primate brain, one that could shape later-life vulnerability to cognitive decline and neurodegeneration. By integrating data across molecular layers, I identify candidate regulatory networks – linking DNA methylation at *cis*-regulatory elements to age-associated gene expression changes, and potential miRNA-mediated post-transcriptional control to transcriptional remodeling – that may play important roles in shaping hippocampal aging. Several of these candidate regulatory relationships implicate genes and pathways with direct relevance to human neurodegenerative disease.

From a biological anthropology perspective, characterizing the molecular landscape of normal brain aging in a nonhuman primate provides an essential comparative baseline for understanding what is shared and what is derived in human brain aging. The regulatory processes identified in this dissertation – shaped across the lifespan by both biology and environment – highlight important genes and pathways in primate aging that may show divergent regulation in humans, potentially underlying our increased vulnerability to neurodegenerative disease. This dissertation includes supplemental files in the form of tables.

This dissertation includes previously published co-authored material.

ACKNOWLEDGMENTS

This dissertation was made possible by the contributions and support from many people who inspired me and aided in my development as a researcher. I would like to thank my dissertation committee including Kirstin Sterner, Nelson Ting, Stephen Frost and William Cresko. A special thanks to my advisor Kirstin who has taught me so much about what it means to be a scientist. Your guidance has been invaluable.

I would also like to thank my collaborators who gave me critical feedback on this work and helped make this work possible including Lucia Carbone, Steven Kohama, Sam Peterson, Marina Watowich, Noah Snyder-Mackler, and Kenneth Chiou. I would also like to acknowledge my funding sources including NSF DDRIG Grant (BCS-2217961), UO and OHSU Collaborative Seed Grant program, NIA/NIH funding sources and by ONPRC support (P51 OD 011092). This work benefitted from access to the University of Oregon's high performance computing cluster Talapas. Some original figures were created using BioRender.

I also want to acknowledge the friends and family members that helped me complete this dissertation. Thank you to Enrique Gomez, Tabor Whitney and Samantha Queeno for being both my academic peers and support during this process. Your friendship during this period was essential and helped me become a better researcher. Thank you to my family members for supporting me including my parents Jerry and Kristie, my sisters Josie and Sophie and my husband Ryan. Lastly, thank you to my grandparents Jacque and Verdun who always believed in the power of education and supported anyone pursuing it.

DEDICATION

To my parents Jerry and Kristie, my sisters Josie and Sophie and my husband Ryan.

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CHAPTER I

INTRODUCTION

Aging is a universal and multifaceted biological process characterized by the progressive decline of physiological function over time and an increased risk of disease and death. Despite its ubiquity, defining and studying aging has been historically challenging due to its complexity, its variability across biological scales, and the absence of a single unifying mechanism (Balcombe and Sinclair 2001; López-Otín et al. 2023; Metcalf et al. 2026). Rather than arising from a singular cause, aging is now understood as the cumulative outcome of multiple interacting biological processes that operate across molecular, cellular, and organismal levels (Weinert and Timiras 2003).

A major advance in conceptualizing these processes is the framework of the *Hallmarks of Aging*, which describes a set of conserved and interconnected mechanisms that drive age-associated decline (López-Otín et al. 2023). These hallmarks include primary drivers of damage such as genomic instability and mutation, telomere attrition, loss of proteostasis, disabled macroautophagy and epigenetic alterations; antagonistic responses such as mitochondrial dysfunction, deregulated nutrient sensing and cellular senescence; and integrative hallmarks including chronic inflammation, dysbiosis, stem cell exhaustion, and altered intercellular communication. While primary hallmarks directly disrupt the regulation, maintenance, and expression of the genome, the downstream antagonistic and integrative responses ultimately drive functional decline and increased disease susceptibility. Importantly, many age-related diseases, including neurodegenerative disorders, can be understood as emergent consequences of

these interacting hallmarks rather than independent processes (Kennedy et al. 2014; López-Otín et al. 2023).

Although the hallmarks framework defines common mechanisms of aging, these processes do not manifest uniformly. The pace and phenotypes of aging vary widely across species (Buffenstein 2009; Reichard 2016; Roach 2016), individuals (Lemaître et al. 2013; Rando and Wyss-Coray 2021; S. Li et al. 2020; Sayer and Cooper 2006), and across tissues within the same organism (Brinkmeyer-Langford et al. 2016; Yamamoto et al. 2022; Cai et al. 2023; Yang et al. 2023; Sadoughi et al. 2025). This heterogeneity suggests that aging is not simply the accumulation of damage, but also reflects differences in how biological systems regulate, respond to, and compensate for that damage over time. Understanding the molecular basis of this variation remains a central challenge in aging biology. Across species and tissues, variation in the pace of aging reflects differences in how effectively regulatory systems are maintained over time. Recent advances, including epigenetic clocks and multi-omics analyses, have demonstrated that aging is marked by tissue-specific patterns of regulatory change (Horvath 2013; Zampieri et al. 2015; Armstrong et al. 2017; Fransquet et al. 2019; Guevara and Lawler 2018; Y.-M. Wang et al. 2025; Zhang et al. 2021; Navandar et al. 2024; Qi et al. 2022; Pereira Fernandes et al. 2018) that do not follow simple linear trajectories (Shen et al. 2024; T. J. Anderson et al. 2025; Vershinina et al. 2021; X. Ma et al. 2026).

At the molecular level, many hallmarks of aging converge on the regulation of gene expression. Epigenetic alterations, transcriptional dysregulation, and disruptions in post-transcriptional control can directly influence how genetic information is maintained and utilized over time (Y. Li and Tollefsbol 2016; López-Otín et al. 2023). These processes are not static; rather, they are dynamic and shaped by both intrinsic programs and environmental exposures

across the lifespan (Goldman and Sterner 2023). As a result, age-related changes in gene regulation provide a mechanistic link between molecular aging processes and variability in phenotypic outcomes, including differences in disease susceptibility across tissues and species (J. Zhao et al. 2024; J. Guo et al. 2022; Arif et al. 2025). Importantly, these regulatory layers do not act in isolation; they interact with one another and with internal and external environments to shape aging trajectories.

An important feature of molecular aging is the progressive loss of regulatory precision, often described as regulatory "drift." This phenomenon is characterized by increased variability in gene expression, erosion of epigenetic patterning, and disruption of post-transcriptional mechanisms that normally maintain cellular identity (Oh et al. 2016; Signor and Nuzhdin 2018). Regulatory drift maps onto several hallmarks of aging, including epigenetic alteration, altered intercellular communication, and deregulated nutrient and gene-sensing pathways, and may represent a unifying mechanism linking molecular dysfunction to cellular and tissue-level decline (Y. Li and Tollefsbol 2016; Oh et al. 2016; López-Otín et al. 2023). Importantly, by eroding the regulatory precision that normally buffers cells against damage, these changes may also increase vulnerability to age-related diseases, including neurodegenerative disorders (Alashwal et al. 2012; Y. Li and Tollefsbol 2016; Marzullo et al. 2023).

One organ particularly vulnerable to age-related changes in gene regulation is the brain, which relies on tightly coordinated molecular and cellular processes to support cognition, learning, and memory (R. Peters 2006; Finch and Austad 2012). Brain aging is associated with multiple hallmark-related processes, including genomic instability, epigenetic alterations, mitochondrial dysfunction and altered intercellular communication, which manifest as synaptic remodeling, glial activation, chronic low-grade inflammation and widespread transcriptional

change (Finch and Austad 2012; Glisky 2007; Gallagher and Rapp 1997; Moss et al. 1999). These processes do not affect all brain regions equally. The hippocampus, a key structure for learning and memory (Anand and Dhikav 2012), is particularly vulnerable to aging-related decline (R. Peters 2006). In this region, age-associated dysfunction is thought to arise from the interaction of multiple hallmarks: for example, epigenetic alterations and transcriptional dysregulation can impair neuronal plasticity, while mitochondrial dysfunction and altered intercellular communication contribute to synaptic loss and neuroinflammation (Lazarov et al. 2024; Baier et al. 2025; Llanquinao-Sandoval et al. 2025; Polzer et al. 2025; M. Sun et al. 2026). Despite these insights, the regulatory features underlying hippocampal aging, particularly how multiple layers of gene regulation change and interact over time, remains incompletely understood.

This dissertation addresses these gaps by examining age-related changes in gene regulation in the hippocampus of rhesus macaques across the adult lifespan. Specifically, it focuses on how multiple molecular layers – including gene expression, DNA methylation (DNAm), and microRNA (miRNA) expression – change with age and interact with one another. By integrating these regulatory layers, this work aims to better understand how core hallmarks of aging manifest at the molecular level in the brain and contribute to variability in aging trajectories. Collectively, this work provides a framework for understanding how gene regulation changes with age in the primate brain and offers insight into the mechanisms that may underlie differential vulnerability to cognitive decline and neurodegenerative disease observed in humans.

1.1 Animal models of human brain aging: from rodents to nonhuman primates

Rodents have provided foundational insights into the biology of aging and remain indispensable for experimental manipulation and mechanistic discovery (Colman 2018; Nadon 2006). However, their relatively short lifespans and accelerated developmental trajectories limit their ability to capture key features of primate brain aging (Finch and Austad 2012; Chiou et al. 2020; Colman 2018). Rodents lack the prolonged postnatal brain maturation characteristic of primates – a developmental phase integral to establishing synaptic connectivity and cognitive capacity (Gogtay et al. 2004; Tau and Peterson 2010). Differences in biological timescales between short-lived and long-lived species further shape how aging processes unfold across the lifespan, influencing when and how regulatory systems begin to deteriorate with fluctuations in early life experience having greater long-term impacts in short-lived species compared to longer-lived species (Kuzawa and Thayer 2011). As a result, rodent models may incompletely represent molecular processes that are particularly relevant to aging in the primate brain.

Primates exhibit distinct life-history characteristics that further differentiate them from short-lived model organisms. Compared to rodents, primates display prolonged juvenile development, high encephalization, and relatively slow rates of senescence – traits that are especially pronounced in humans (Kirkwood and Westendorp 2001; Bogin 1999; Leonard et al. 2007; Dunbar and Shultz 2007; Pignolo 2019). These extended lifespans amplify the cumulative physiological burden of aging and may disproportionately impact energetically expensive tissues such as the brain, where long-term maintenance of complex cellular and regulatory systems is required (Mohan et al. 2016; A Peters et al. 1998; Bauernfeind and Babbitt 2020).

Nonhuman primates therefore provide a critical complementary avenue for studying brain aging in a biologically relevant context. Among these, the rhesus macaque (*Macaca mulatta*) is

especially well suited for investigating the molecular basis of aging in the brain. Rhesus macaques share a high degree of genomic and physiological similarity with humans and exhibit complex cognitive and social behaviors (Chiou et al. 2020; Ingram et al. 2001; Roth et al. 2004). They also display key features of primate life history, including prolonged development and gradual senescence relative to rodents, while maintaining a compressed lifespan making them a powerful system for investigating the mechanisms of primate brain aging (Chiou et al. 2020). Rhesus macaques typically reach sexual maturity between approximately 2.5 and 4.5 years of age and have a median lifespan of ~25–27 years in captivity, with shorter lifespans in the wild (Lewis and Prongay 2015; Chiou et al. 2020).

In the brain, rhesus macaques recapitulate many features observed in human brain aging, including age-related changes in hippocampal structure, synaptic organization and gene expression (Alan Peters and Kemper 2012; Zhang et al. 2021). However, they fail to develop the full neuropathological profile characteristic of human neurodegenerative diseases such as Alzheimer's disease (Walker and Jucker 2017). This distinction provides a unique opportunity to differentiate molecular features of normative brain aging from those associated with disease vulnerability in humans.

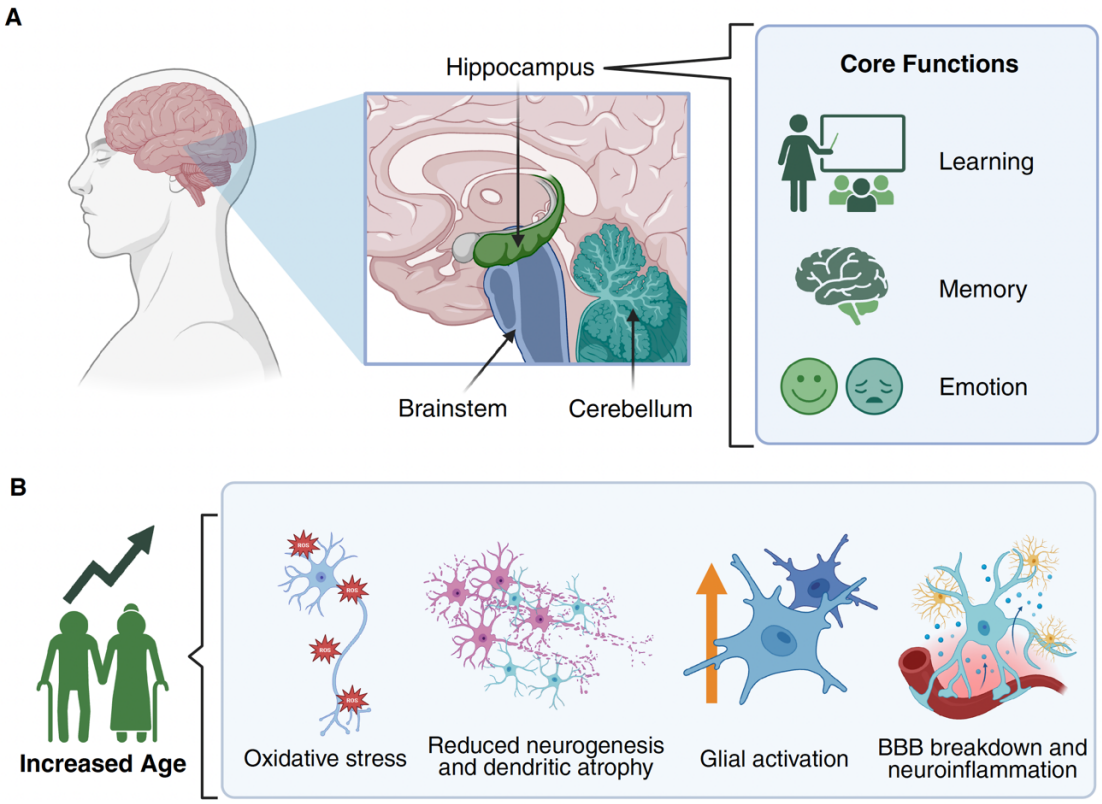
1.2 The hippocampus, biology and aging

The hippocampus is a central hub for learning, memory, and emotion (**Fig. 1.1A**), and is among the brain regions most sensitive to the effects of aging (Anand and Dhikav 2012; R. Peters 2006). Located within the medial temporal lobe, it plays a critical role in episodic memory formation and spatial navigation (El-Falougy and Benuska 2006). Structurally, the hippocampus comprises several anatomically and functionally distinct subfields – including the dentate gyrus,

CA1-CA3 regions, and subiculum – which differ in connectivity, cellular composition, and vulnerability to aging-related change (de Flores et al. 2015). Despite this regional specialization, convergent patterns of age-associated decline are observed across the hippocampus, allowing it to be studied as a functionally integrated system in the context of aging.

Across the lifespan, hippocampal function depends on high levels of synaptic plasticity, ongoing neurogenesis, and tightly coordinated interactions among neuronal, glial, and vascular systems. This capacity for plasticity, while essential for encoding new experiences, also renders the hippocampus particularly sensitive to age-related damage (Bartsch and Wulff 2015). With advancing age, the capacity to maintain and regulate these plastic processes is progressively diminished and decline is accompanied by reduced neurogenesis, dendritic atrophy, increased oxidative stress, heightened glial activation, chronic neuroinflammation, and early disruption of blood–brain barrier integrity (**Fig. 1.1B**) (Finch and Austad 2012; Bettio et al. 2017; Montagne et al. 2015). Together, these changes impair synaptic function ultimately contributing to age-related cognitive decline.

Fig. 1.1 (next page) The hippocampus is a brain region particularly susceptible to aging-related processes. A) Anatomical localization of the human hippocampus within the medial temporal lobe within context of neighboring brain regions (e.g., brainstem and cerebellum). The hippocampus, highlighted in green and shown in sagittal view, plays a critical role in shaping learning, memory and emotion. B) Key cellular and molecular hallmarks of hippocampal aging. Hallmarks of aging in this tissue include increased oxidative stress, reduced neurogenesis, dendritic atrophy, heightened glial activation, and blood-brain barrier (BBB) breakdown accompanied by neuroinflammation. Together, these processes contribute to altered neuronal function, impaired plasticity and increased vulnerability to cognitive decline during aging



These cellular changes are paralleled by shifts in primate hippocampal cell population dynamics, including reduced proliferative capacity of neural progenitors, heightened inflammatory responses in microglia and oligodendrocytes, and altered endothelial signaling that collectively contribute to a less supportive microenvironment for neurogenesis (Zhang et al. 2021). At the molecular level, hippocampal aging is accompanied by coordinated changes in gene expression that reflect these underlying cellular processes. Transcriptomic studies consistently report age-associated upregulation of immune and inflammatory pathways alongside downregulation of metabolic, synaptic, and plasticity-related gene networks. These patterns have been observed in the hippocampus of aging rodents (C. K. Lee et al. 2000; Rowe et al. 2007), rhesus macaques (T. J. Anderson et al. 2025; Blalock et al. 2010), and in hippocampal and

neocortical tissue from aging humans (T. Lu et al. 2004; McGeer and McGeer 2004; Parachikova et al. 2007).

Comparative studies further indicate that the hippocampus is a region in which humans diverge from other primates in vulnerability to neurodegenerative disease. With advanced age, humans and several nonhuman primate species develop amyloid-β deposition and tau pathology within the hippocampus; however, in macaques and chimpanzees these features typically emerge later in life, occur at lower abundance, and are not accompanied by the extensive neuronal loss or severe cognitive impairment that define Alzheimer's disease in humans (Alan Peters and Kemper 2012; Rosen et al. 2008). Although early work suggested that nonhuman primates were largely resistant to Alzheimer's-like pathology, it is now recognized that such lesions do occur but are generally restricted to very old individuals and differ qualitatively from human disease (Walker and Jucker 2017). This dissociation suggests that while fundamental hallmarks of brain aging are conserved across primates, species-specific differences in regulatory responses, cellular resilience, or network-level compensation may determine whether aging-associated changes progress to overt neurodegeneration.

Despite the hippocampus's central role in cognition and its clear relevance to aging-related disease, gene regulatory signatures of hippocampal aging in nonhuman primates remain incompletely characterized. This gap is particularly pronounced during midlife, a period recognized as critical for shaping late-life brain aging trajectories and disease risk (Schubert et al. 2019; Debette et al. 2011; Tolppanen et al. 2012; Hatton et al. 2018; Finch 1991; Mann 1997; Coleman et al. 1990). Few studies have examined age-related changes across multiple layers of gene regulation within the same individuals across this window. Given that hippocampal function is tightly coupled to activity-dependent gene expression and plasticity, understanding

how epigenetic, transcriptional and post-transcriptional regulatory mechanisms change with age – and how they relate to core hallmarks such as epigenetic alterations, inflammation and altered intercellular communication – is essential for determining how this region adapts, or fails to adapt, to the cumulative challenges of aging.

1.3 Gene regulatory mechanisms in hippocampal aging

1.3.1 Overview of multi-layer gene regulation

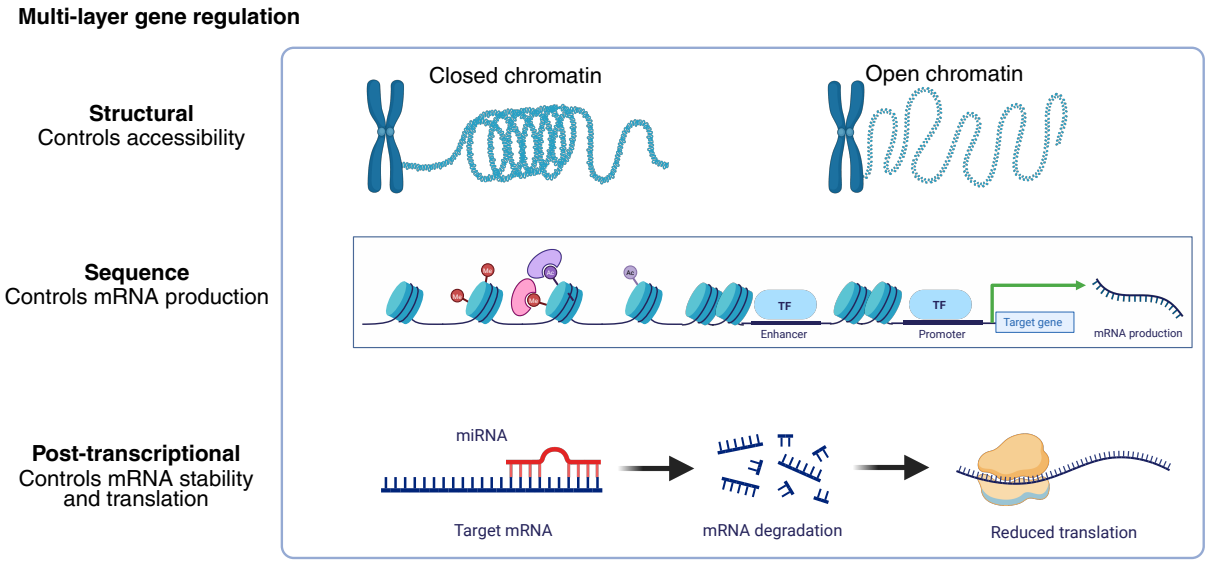
Gene expression is the process through which information encoded in DNA is translated into proteins that ultimately shape phenotype. The central dogma of molecular biology describes this flow of information from DNA to RNA to protein (Crick 1970); however, this process is not linear or uniform. Instead, gene expression is dynamically regulated at multiple levels, allowing precise control over when, where, and how much a gene product is produced.

To produce a gene product, multiple conditions must be met: the chromatin surrounding a gene must be accessible, the necessary transcriptional machinery must be recruited to initiate RNA synthesis, and the resulting transcripts must be properly processed and translated. Each of these steps represents a point at which gene expression can be modulated, allowing responsiveness to developmental cues, environmental signals, and physiological demands.

Gene regulation can therefore be understood as a multi-layered process involving interacting regulatory systems that operate across chromatin structure, genomic sequence, and post-transcriptional (i.e., transcript stability) levels (**Fig. 1.2**). At the structural level, chromatin accessibility and the spatial organization of DNA within the nucleus determines whether genes and *cis*-regulatory elements are available for transcription, with histone modifications shaping this regulatory landscape (Kouzarides 2007; Strahl and Allis 2000; Furlong and Levine 2018;

Rowley and Corces 2018). At the sequence level, gene expression is controlled by the coordinated activity and interaction of transcription factors and *cis*-regulatory elements, including enhancers and promoters, which together regulate the production of messenger RNA (mRNA) transcripts. DNAm – the addition of a methyl group to cytosine residues, most commonly at CpG dinucleotides, though non-CpG methylation is also prevalent in the brain – can further influence this process at regulatory elements by modulating transcription factor binding and local regulatory activity, thereby silencing or activating gene expression depending on genomic context (L. D. Moore et al. 2013; Héberlé and Bardet 2019; Schoenfelder and Fraser 2019; Stadhouders et al. 2019). At the post-transcriptional level, regulatory mechanisms including miRNAs, RNA-binding proteins and other small noncoding RNAs modulate transcript stability and translation, fine-tuning protein output through mRNA degradation and translational repression (Bartel 2018).

Fig. 1.2 (next page) Multi-layer gene regulation shapes hippocampal aging. Overview of hierarchical gene regulatory control across structural, sequence, and post-transcriptional (i.e., transcript stability) layers. At the structural layer, chromatin accessibility (closed versus open) and the physical organization of DNA within the nucleus determine whether genes and *cis*-regulatory elements are available for transcription, with histone modifications shaping this regulatory landscape. At the sequence level, elements such as DNAm, transcription factors (TFs) and other *cis*-regulatory elements (including enhancers and promoters) modulate mRNA production in both activating and repressive manners. At the post-transcriptional (i.e., transcript stability) level, miRNAs and other regulatory mechanisms modulate transcript stability and translation, including through mRNA degradation and translational repression. These regulatory layers do not strictly operate in a unidirectional manner: chromatin state can influence miRNA production and transcription factor access, transcriptional activity can reshape chromatin organization, and miRNAs can regulate transcripts encoding epigenetic modifiers and transcriptional regulators



These regulatory layers do not act independently. Epigenetic states influence transcription factor binding and transcriptional output, transcriptional activity can in turn reshape chromatin organization and post-transcriptional regulators such as miRNAs can target transcripts encoding epigenetic modifiers and transcription factors. Through these interactions, gene regulatory systems form interconnected feedback networks that stabilize gene expression while remaining responsive to internal and external signals (Rowley and Corces 2018; Bartel 2018; Benayoun et al. 2015; Bartel 2004; Danka Mohammed et al. 2017).

Importantly, these regulatory processes provide a major source of biological variation, integrating genetic background with environmental exposures across the lifespan. Aging is associated with a progressive disruption of this coordinated regulation, leading to reduced regulatory fidelity, increased transcriptional variability, and epigenetic drift – features that map directly onto core hallmarks of aging (López-Otín et al. 2023).

Understanding hippocampal aging therefore requires an integrative framework that captures how these regulatory systems interact and how their coordination deteriorates over time.

This dissertation adopts a multi-omic approach to characterize epigenetic, transcriptional and post-transcriptional regulation in the primate hippocampus, providing insight into how changes across regulatory layers shape the molecular trajectory of brain aging. The following sections review key findings from rodent, nonhuman primate and human studies of hippocampal aging at each of these regulatory levels (epigenetic, transcriptional, and post-transcriptional) before considering how an integrative, multi-omic framework can capture their interactions.

1.3.2 DNA methylation and chromatin remodeling in hippocampal aging

Epigenetic alterations, including changes in DNAm and histone modifications, are a defining feature of aging and contribute to the progressive destabilization of gene regulatory systems over time (Sen et al. 2016; Seale et al. 2022; López-Otín et al. 2023). With age, the epigenome becomes increasingly unstable – a phenomenon often referred to as epigenetic drift. This drift is characterized by stochastic and locus-specific changes in DNAm, shifts in histone modification patterns and large-scale chromatin remodeling (Horvath and Raj 2018; Seale et al. 2022). While many of these modifications are potentially reversible, their cumulative effects can disrupt transcriptional control and promote cellular dysfunction (Xu 2015; Unnikrishnan et al. 2019). Rather than reflecting a single process, epigenetic drift arises from imperfect maintenance of epigenetic marks over time, leading to increased variability in the methylome and a decline in the coordinated regulation of gene networks (M. J. Jones et al. 2015; Issa 2014; Hernando-Herraez et al. 2019; Fan et al. 2025; Bacalini et al. 2017; Seale et al. 2022).

Rodent studies have been foundational in defining how these changes manifest in the hippocampus. Histone modification patterns – chemical groups added to histone proteins that alter chromatin compaction and gene accessibility – undergo age-dependent remodeling in the

rodent hippocampus. Aging alters the balance between activating and repressive histone marks, such as H3K4me3 and H3K27me3, impairing the induction of plasticity-related genes like *Bdnf* (Palomer et al. 2016). In aged rats, reduced NMDA receptor signaling reduces capacity for chromatin remodeling at *Bdnf* promoters, while environmental enrichment restores these processes and rescues memory performance (Palomer et al. 2016; Morse et al. 2015). At a broader scale, aging is also associated with loss of constitutive heterochromatin and redistribution of chromatin states, reflecting large-scale reorganization of genome architecture (Pérez et al. 2024).

Although global DNAm levels remain relatively stable with age, coordinated changes emerge across the genome in the form of differentially methylated regions (DMRs), particularly within intergenic and intronic regions (Masser et al. 2017; Unnikrishnan et al. 2018). These patterns have been characterized using genome-wide approaches such as whole-genome bisulfite sequencing (WGBS) and reduced-representation bisulfite sequencing (RRBS), two common approaches which enable high-resolution mapping of DNAm across the genome. Age-associated changes include both gains and losses of methylation, with some evidence for sex-specific effects (Masser et al. 2017; Chinn et al. 2021). For example, female mice often show greater age-related methylation changes in inflammatory gene networks compared to males (Chinn et al. 2021). Notably, genome-wide methylation analyses reveal that environmental enrichment can partially reverse age-associated loss of methylation at sites involved in neuroplasticity and neurogenesis (Zocher et al. 2021), demonstrating that the aging methylome retains a degree of malleability in response to environment.

Paired methylome-transcriptome analyses further demonstrate that the relationship between DNAm and gene expression during aging is context-dependent and varies by genomic

location. For example, methylation at promoter regions is often associated with transcriptional repression, whereas gene body methylation can show more complex or even positive associations with expression (P. A. Jones 2012; L. D. Moore et al. 2013). In the hippocampus, genes with higher levels of methylation in younger adult individuals tend to increase in expression with age, while those with lower initial methylation tend to decline (Hadad et al. 2019). These findings suggest that epigenetic states established earlier in adulthood can shape transcriptional trajectories during aging. Consistent with this, age-related methylation changes at activity-dependent genes are associated with reduced transcriptional plasticity and impaired memory formation (M. R. Penner et al. 2011; M. Penner et al. 2016).

In contrast to the extensive rodent literature, studies of epigenetic aging in primate brains remain comparatively limited, largely due to challenges in obtaining well-characterized tissue across the lifespan. Nevertheless, emerging datasets are beginning to reveal both conserved and divergent features of epigenetic aging across mammals. In the brain, DNAm is preferentially localized to regulatory elements such as enhancers that shape neuronal identity and activity, a pattern that is broadly conserved across mammals (Lister et al. 2013). Along with this, single-nucleus transcriptomic analyses in rhesus macaques show that aging in the hippocampus is associated with DNA damage, heterochromatin erosion, increased inflammation, and loss of proteostasis (Zhang et al. 2021). While these patterns parallel those observed in rodents, their onset and progression appear shifted later in the lifespan, consistent with the slower pace and extended duration of aging in primates (Zhang et al. 2021).

Recent large-scale single-nucleus multi-omic atlases of the adult rhesus macaque brain integrate transcriptomic (snRNA-seq) and chromatin accessibility (snATAC-seq) data across 30 regions, including the hippocampus, identifying over one million candidate *cis*-regulatory

elements and mapping cell-type–specific enhancer and promoter landscapes (Chiou et al. 2023). These datasets provide crucial context for interpreting age-related chromatin remodeling, revealing that transcription factor motif accessibility is tightly coupled to gene expression, reinforcing a regulatory cascade from chromatin state to transcriptional output. Complementing these cross-sectional resources, the development of macaque-specific epigenetic clocks demonstrates that methylation dynamics at specific CpG sites are predictive of biological age, providing a quantitative framework for studying primate epigenetic aging (Horvath et al. 2021; Goldman et al. 2022; Sadoughi et al. 2025).

Despite these advances, multi-layer studies of hippocampal methylation and chromatin accessibility across the primate lifespan remain limited. Current evidence (e.g., heterochromatin erosion, enhancer remodeling, and epigenetic drift) suggests that the macaque hippocampus undergoes similar epigenetic aging processes as rodents, but over a longer timescale and with delayed onset.

Human studies further support the conserved nature of epigenetic aging while highlighting region-specific and disease-associated variation. DNAm changes in the aging human brain are highly regionalized, with cortical and hippocampal areas showing pronounced remodeling (Pellegrini et al. 2021). Many of these changes parallel those observed in Alzheimer's disease, consistent with the concept of accelerated epigenetic aging in neurodegeneration (Pellegrini et al. 2021). In addition, age- and disease-associated shifts in DNAm have been observed in the hippocampus and are associated with pathological features such as amyloid accumulation and neurofibrillary tangles (Chouliaras et al. 2013). These patterns are consistent with increasing entropy of the methylome, a hallmark of epigenetic drift, that likely contributes to the breakdown of neuronal identity and homeostasis (Jeong et al. 2025).

Together, these findings indicate that epigenetic aging in the hippocampus is characterized by increasing variability in DNAm, disruption of chromatin organization, and altered regulation of genes involved in neuronal function and plasticity. These suggest that primary (chronological, intrinsic epigenetic change) and secondary hallmarks of aging (disease- or environment-driven remodeling) are closely intertwined (Seale et al. 2022; López-Otín et al. 2023). At the same time, a subset of DNAm changes occurs in reproducible patterns that can be captured by epigenetic clock models, which accurately predict chronological age across tissues and species (Sadoughi et al. 2025; Horvath and Raj 2018; Unnikrishnan et al. 2019). This coexistence of stochastic variation and predictable change highlights a central feature of aging biology: while core molecular processes are conserved, their regulation and manifestation vary across biological contexts. In the hippocampus, such differences likely reflect how gene regulatory systems are maintained and remodeled over time. While the overall features of epigenetic aging are conserved, variation in the timing and consequences of these changes may contribute to the heightened vulnerability of the human hippocampus to neurodegenerative disease (Bartsch and Wulff 2015).

1.3.3 Transcriptional signatures of hippocampal aging

Aging is accompanied by profound transcriptional reorganization across multiple brain regions, and the hippocampus appears noticeably susceptible to these changes (T. Lu et al. 2004; Yankner et al. 2008; Finch and Austad 2012). Across mammals, studies consistently show that advancing age alters gene expression, reflecting a breakdown in the tight molecular control that supports neuronal function, plasticity, and cellular homeostasis (T. Lu et al. 2004; Finch and Austad 2012; Ianov et al. 2017; González-Velasco et al. 2020; Zhang et al. 2021).

Transcriptomic studies in rodents, nonhuman primates, and humans collectively reveal recurring signatures of aging, including increased expression of inflammatory and stress-response pathways, diminished expression of genes involved in synaptic signaling and metabolism, and a general rise in transcriptional noise that signals reduced regulatory precision (Zhang et al. 2021; Ianov et al. 2017; González-Velasco et al. 2020; Chiou et al. 2022).

Rodent hippocampal transcriptomics have provided foundational insight into the molecular hallmarks of brain aging. In the rodent hippocampus, age-related upregulation of immune and inflammatory genes occurs alongside downregulation of synaptic and calcium-signaling genes, changes that correlate with poorer memory performance on hippocampus-dependent tasks (Ianov et al. 2017; Smith et al. 2020). Mouse studies using bulk RNA-seq and ribosome profiling similarly demonstrate increased expression of immune and glial genes and decreased expression of transcripts linked to mitochondrial and synaptic function, with age-dependent divergence between transcriptional and translational control (Winsky-Sommerer et al. 2023). Single-cell RNA-seq and spatial transcriptomic analyses have refined this picture, showing that the aging mouse hippocampus exhibits reduced expression of neurogenesis- and synapse-associated genes, increased neuroinflammatory activity, and reorganization of cellular niches, particularly around the dentate gyrus where neural stem cell activation declines (Conacher et al. 2025; Wu et al. 2025). Complementary proteomic/transcriptomic work confirms two dominant molecular axes of aging in the rodent hippocampus with upregulation of inflammation-related pathways and downregulation of RNA metabolism and protein synthesis (Pérez et al. 2024). These changes were shown to be partially reversible by environmental enrichment.

Studies in macaques reveal aging patterns that more closely mirror those observed in humans, suggesting their importance as a more translatable model for human brain aging. Single-nucleus transcriptomic profiling of the macaque hippocampus demonstrates broad age-related changes spanning multiple cell types. In older individuals (generally >18 years), neurons and neural progenitors show reduced expression of genes linked to neurogenesis, differentiation, and synaptic plasticity, while glial and endothelial populations display elevated inflammatory signatures (Zhang et al. 2021; W. Wang et al. 2022). These shifts coincide with increased DNA damage and heterochromatin erosion (Zhang et al. 2021), suggesting that both transcriptional and epigenetic regulation deteriorate with age. Interestingly, macaques maintain higher expression of neurogenic and plasticity-associated genes into adulthood compared to humans, a pattern that has been suggested to contribute to their relative resistance to overt neurodegeneration despite shared aging-associated molecular changes (Y. Zhu et al. 2018).

Across multiple brain regions, including the hippocampus, bulk and single-nucleus RNA-seq data from rhesus macaques reveal extensive age-associated transcriptional remodeling that parallels human trajectories (Chiou et al. 2022). These changes include downregulation of neuronal signaling and synaptic genes and upregulation of immune and stress-response pathways, accompanied by increased transcriptional variability – a hallmark of declining regulatory control. Moreover, environmental and social factors may modulate these molecular trajectories, with this study finding that high-ranking female macaques exhibit "younger" hippocampal transcriptomic signatures, suggesting that social environment can influence molecular aging rates (Chiou et al. 2022).

Human studies, although limited, extend these findings and reveal both conserved and region-specific molecular signatures of aging in the hippocampus. Similar to other mammals,

integrative analyses of large-scale RNA-seq and microarray datasets show that the human hippocampus undergoes pronounced downregulation of synaptic, neurotrophic, and calcium-signaling genes, alongside upregulation of immune and metabolic stress pathways (González-Velasco et al. 2020). These transcriptional shifts are not unique to the hippocampus; studies in other human brain regions (e.g., prefrontal cortex) also demonstrate coordinated transcriptional cascades across the lifespan, with inflammatory and glial genes (e.g., *TLR4*, *IL1R1*, *NFKB1*) becoming increasingly expressed during aging as synaptic and neurotrophic genes (e.g., *BDNF*, *SYN*, *DBN1*) decline (Primiani et al. 2014). These results suggest that aging reorganizes gene regulatory networks that maintain neuronal health and synaptic connectivity, shifting the molecular landscape of the hippocampus (and brain more generally) toward an inflammatory and metabolically stressed state that may be particularly exacerbated in humans (T. Lu et al. 2004; Finch and Austad 2012; González-Velasco et al. 2020).

Taken together, rodent and primate studies converge on a common picture: hippocampal aging is characterized by both coordinated shifts in gene expression and a progressive loss of regulatory precision, reflected in declining coordination among gene networks that support neurogenesis, synaptic function, and metabolic stability. At the same time, immune activation, stress-response signaling, and glial remodeling become increasingly dominant components of the transcriptome. Although these transcriptional trends appear broadly conserved across species, their timing and magnitude vary, with nonhuman primates exhibiting a delayed emergence of aging-associated transcriptional signatures and, in some cases, prolonged maintenance of neuronal and plasticity-related gene expression relative to shorter-lived mammals (Zhang et al. 2021; Conacher et al. 2025). Advances in transcriptomic technologies, particularly single-cell and spatial approaches, have made it clear that aging does not simply alter bulk gene expression

but remodels the cellular and regulatory architecture of the hippocampus in complex, cell-type-specific ways (Huang et al. 2025). Together, these findings highlight the importance of examining transcriptional remodeling within an integrated, multi-omic framework to understand how changes in RNA abundance, chromatin state, and post-transcriptional regulation collectively shape the molecular trajectory of brain aging.

1.3.4 Post-transcriptional regulation and small RNAs in hippocampal aging

Post-transcriptional regulation represents a critical layer of molecular control that links RNA synthesis to protein production. Examples of post-transcriptional regulation include the processing, alternative splicing, editing, and transport of RNA, as well as mechanisms that support transcript stability or degradation. These mechanisms determine how RNA transcripts are processed, stabilized, and translated, thereby shaping how much protein is ultimately produced from a given gene. In the hippocampus, where neuronal function depends on the rapid, localized translation of synaptic mRNAs, post-transcriptional fidelity is essential for maintaining plasticity and cognitive performance (Fernandez-Moya et al. 2014; Aakalu et al. 2001). Increasing evidence across mammals indicates that aging disrupts this fine-tuned regulatory network, leading to altered isoform diversity, translational imbalance, and reorganization of small RNA-mediated control systems that together reshape the hippocampal molecular landscape (Danka Mohammed et al. 2017; Winsky-Sommerer et al. 2023; Stilling et al. 2014; Danka Mohammed et al. 2016; Alberro et al. 2024).

At the level of mRNA processing and translation, aging is accompanied by widespread disruption of post-transcriptional mechanisms. In the aging mouse hippocampus, transcriptional upregulation of immune and inflammatory pathways is mirrored by translational activation,

whereas genes involved in mitochondrial function, calcium signaling, and the cell cycle display discordant expression patterns indicating impaired translational efficiency and a partial decoupling of the transcriptome and translatome (Winsky-Sommerer et al. 2023). Similarly, age-dependent increases in alternative splicing and RNA editing are observed, particularly in genes related to synaptic function, RNA-binding proteins, and dendritic signaling (Stilling et al. 2014). Collectively, these studies highlight that post-transcriptional remodeling is not merely a downstream reflection of transcriptional change but a distinct axis of molecular aging that contributes to loss of proteostasis, a core hallmark of aging (López-Otín et al. 2023) that may make the hippocampus more susceptible to age-related changes (Bettio et al. 2017).

Among the most pervasive mediators of post-transcriptional regulation are small noncoding RNAs, particularly miRNAs, which serve as critical modulators of gene expression in the brain (Bartel 2018, 2004; Somel et al. 2013). Mature miRNAs are ~22 nucleotides in length and regulate translation through several mechanisms including: 1) they can bind near the 3′ untranslated region of target mRNAs to promote transcript destabilization and degradation; 2) they can cause cleavage of the mRNA strand; or 3) they can prevent ribosomal binding and thereby block translation (Bartel 2018, 2004). Through these processes, miRNAs fine-tune protein synthesis, acting as molecular regulators that balance mRNA abundance with functional protein output. Because individual miRNAs can target hundreds of transcripts, often within interconnected pathways, they exert broad influence over neuronal homeostasis and synaptic plasticity (Somel et al. 2013). In the hippocampus – where local translation of dendritic mRNAs underlies long-term potentiation and memory formation – miRNAs play an essential role in regulating activity-dependent protein synthesis, making them key components of neuronal and cognitive stability (Fernandez-Moya et al. 2014; Aakalu et al. 2001).

A growing body of evidence demonstrates that miRNA expression is dynamically remodeled during aging and contributes to many hallmarks of brain aging, including altered cognition, inflammation, neuroprotection, lipid metabolism, mitochondrial function, and lifespan (Danka Mohammed et al. 2017, 2016; Che et al. 2014). Across rodent models, dozens of hippocampal miRNAs show age-related changes that shape pathways central to neuronal and metabolic maintenance (Danka Mohammed et al. 2016; Che et al. 2014; Munkhzul et al. 2023). For instance, the upregulation of *miR-204* suppresses *EphB2* translation, reducing synaptic signaling capacity, while downregulation of miRNAs targeting *APP* and *BACE1* permits amyloidogenic protein accumulation in the hippocampus (Danka Mohammed et al. 2016; Che et al. 2014). These examples illustrate how age-dependent miRNA remodeling influences protein synthesis independently of mRNA abundance, providing a mechanistic link between post-transcriptional dysregulation and neurodegeneration. Notably, miRNA expression is itself regulated by epigenetic mechanisms, including DNAm and chromatin accessibility at miRNA loci and promoters, creating an additional layer of interaction between epigenetic and post-transcriptional regulation. Age-associated changes in chromatin state may therefore indirectly reshape miRNA-mediated control of gene expression, reinforcing cross-layer regulatory drift during aging (Sato et al. 2011; Garg and Cohen 2014).

Beyond neuronal signaling, miRNAs also mediate the interplay between aging, stress, and environment. In the hippocampus of rodents, exposure to physiological or pathological stressors (specifically sepsis) alters miRNA expression in an age- and sex-dependent manner, influencing inflammatory and neuroprotective responses (Rani et al. 2022). Similarly, estrogen-dependent modulation of miRNA networks shapes hippocampal gene regulation across the female lifespan (Rao et al. 2013). These findings align with evidence that miRNA expression

patterns are highly responsive to environmental and metabolic inputs, reflecting ongoing crosstalk between environment and gene regulation during aging (T. Singh and Yadav 2020; Smit-McBride et al. 2018). This regulatory sensitivity may contribute to interindividual variation in cognitive aging and highlight miRNAs as molecular mediators of environment and aging biology.

Although most mechanistic insight into miRNA function in brain aging derives from rodents, comparative studies reveal that miRNA expression is deeply conserved among primates. Analyses in humans and macaques indicate that miRNA expression patterns evolve more slowly than those of mRNAs and are tightly coupled to developmental timing and lifespan (Somel et al. 2010). This conservation within primates, and the divergence of primate miRNA profiles from those of shorter-lived mammals, suggests that primate-specific miRNA regulatory networks may contribute to the distinct trajectories of brain aging observed between primates and rodents (Somel et al. 2013). Despite this conservation, data on miRNA-mediated regulation in the primate hippocampus remain scarce representing a major gap in our understanding of post-transcriptional aging in long-lived species.

In humans, previous studies also suggest miRNA remodeling is a conserved and central feature of aging and neurodegeneration (Basak et al. 2016). Early work identified differential expression of *miR-9*, *miR-124*, and *miR-132* in aged and Alzheimer's disease hippocampus (Lukiw 2007; Llorens et al. 2017), consistent with progressive loss of post-transcriptional control in neuronal networks. More recent sequencing of human hippocampal small RNAs revealed that a small subset of miRNAs (*hsa-miR-192-5p* and *hsa-miR-3135b*) account for the majority of mRNA-miRNA regulatory interactions distinguishing older adults from centenarians (Alberro et

al. 2024). These findings suggest that a subset of miRNAs may play important roles in shaping hippocampal aging trajectories and modulating extreme longevity.

Taken together, evidence across species demonstrates that post-transcriptional regulation in the hippocampus can become progressively dysregulated with age. The cumulative effects of reduced splicing precision, weakened coupling between transcription and translation, and remodeling of miRNA networks reflect a decline in the molecular fidelity of regulatory systems that sustain neuronal identity and plasticity (Winsky-Sommerer et al. 2023; Stilling et al. 2014; Danka Mohammed et al. 2016; Alberro et al. 2024). Yet, characterization of post-transcriptional aging in primates remains limited.

1.3.5 Integration across molecular layers

Although transcriptional, epigenetic, and post-transcriptional processes can each be studied independently, they are inherently intertwined components of a unified regulatory network. Aging disrupts this coordination, leading to cascading effects across molecular layers that amplify variability and impair cellular homeostasis (López-Otín et al. 2023; Aunan et al. 2016). Epigenetic drift can alter transcription factor binding and chromatin accessibility, reshaping the transcriptome. Age-dependent miRNA remodeling can suppress or derepress genes encoding epigenetic modifiers and transcriptional regulators, which can ultimately erode the regulatory precision that maintains neuronal identity and function (Danka Mohammed et al. 2017; López-Otín et al. 2023; Seale et al. 2022; Somel et al. 2010; Garg and Cohen 2014). Understanding the molecular mechanisms that shape hippocampal aging, therefore, requires an integrative framework that captures how these molecular hierarchies interact rather than treating them as isolated processes.

Emerging multi-omic studies demonstrate the power of such integrative approaches. Integrating and analyzing DNAm, mRNA abundance, and small RNA expression from matched samples has the potential to reveal important cross-layer interactions that shape aging trajectories and points of convergence where dysregulation may drive neurodegenerative vulnerability (Winsky-Sommerer et al. 2023; Ren et al. 2025; Scheidemantel et al. 2025; Pérez-González et al. 2024). Importantly, this approach enables a more mechanistic understanding of how aging phenotypes arise, including identifying regulatory processes that may be responsive to environmental exposures or interventions that slow, accelerate, or potentially reverse aspects of molecular aging. However, comprehensive, lifespan-spanning molecular analyses in primate brain tissue remain scarce. Applying a multi-omic approach in a long-lived primate model such as the rhesus macaque provides a unique opportunity to disentangle the signatures of molecular aging, highlight potential mechanisms of resilience, and bridge the gap between experimental animal models and human neurodegenerative susceptibility.

1.4 Overview of dissertation

Aging of the hippocampus is a central feature of cognitive decline and neurodegenerative vulnerability, yet the molecular mechanisms that regulate hippocampal aging, particularly across multiple layers of gene regulation, remain poorly characterized in long-lived primates. While transcriptional, epigenetic, and post-transcriptional changes have each been studied in isolation, we lack an integrated understanding of how these regulatory layers interact across the lifespan and when critical transitions in regulatory control occur. Multi-omic analyses have shown that molecular aging exhibits nonlinear patterns across molecular data types, suggesting shared regulatory inflection points that may inform on and precede functional decline (Shen et al. 2024;

G.-H. Li et al. 2025). This gap is especially pronounced for midlife, a critical period increasingly argued to shape the trajectory of brain aging (Coleman et al. 1990; Finch 1991; Mann 1997; Debette et al. 2011; Tolppanen et al. 2012; Hatton et al. 2018; Schubert et al. 2019).

The goal of this dissertation is to characterize epigenetic, transcriptional and post-transcriptional signatures of aging in the hippocampus of rhesus macaques across the lifespan using matched biobanked samples. By leveraging a multi-omic framework in a nonhuman primate, this work aims to identify coordinated regulatory shifts, define candidate gene regulatory networks involved in hippocampal aging, and pinpoint critical periods when molecular trajectories shift. Importantly, by characterizing molecular signatures of aging in a large-brain, longer-lived nonhuman primate, this dissertation provides the comparative context needed to identify where and how human brain aging deviates from primate patterns, and why our species has increased vulnerability to neurodegenerative disease. The following is a summary of Chapters 2-5.

Chapter 2 examines patterns of transcriptomic aging in the rhesus macaque hippocampus across the lifespan using bulk RNA-seq. Using autoregressive integrated moving average (ARIMA) modeling, this chapter identifies age-associated gene expression trajectories that fall into four broad classes, including both monotonic and nonlinear patterns. While most age-associated genes exhibit gradual, monotonic changes, a subset shows pronounced nonlinear trajectories with an inflection point centered around ~10 years of age in macaques (approximately ~30 years in humans). These nonlinear patterns highlight midlife as a potential critical window during which hippocampal transcriptional programs reorganize in ways that may shape later aging trajectories and vulnerability to neurodegeneration. I led this research, with co-authorship from Marina M. Watowich, Kenneth L. Chiou, Elisabeth A. Goldman, Sam Peterson,

Jordan A. Anderson, Noah Snyder-Mackler, Lucia Carbone, Steven G. Kohama, and Kirstin N. Sterner.

Chapter 3 characterizes epigenetic aging in the rhesus macaque hippocampus by profiling DNAm across the lifespan using RRBS and integrating these data with transcriptomic results from Chapter 2. This chapter quantifies multiple dimensions of methylation change, including mean methylation shifts, variance changes, and nonlinear methylation trajectories at both CpG- and region-level resolution. In addition, a hippocampus-specific epigenetic clock is developed that accurately predicts chronological age in macaques. Together, these analyses reveal that the aging hippocampal methylome is dominated by epigenetic drift and, importantly, exhibits midlife-associated transitions centered around ~10 years of age, paralleling transcriptomic findings. Integration with gene expression data identifies candidate regulatory regions that may contribute to age-related transcriptional remodeling. I led this research, with co-authorship from Marina M. Watowich, Kenneth L. Chiou, Maili M. Smith, Elisabeth A. Goldman, Sam Peterson, Noah Snyder-Mackler, Lucia Carbone, Steven G. Kohama, and Kirstin N. Sterner.

Chapter 4 focuses on post-transcriptional regulation by characterizing the general small RNA landscape as well as age-associated changes in miRNA expression in the rhesus macaque hippocampus using small RNA-seq. Using ARIMA modeling, this chapter identifies both monotonic and nonlinear miRNA expression trajectories across the lifespan. Consistent with findings from Chapters 2 and 3, nonlinear miRNA trajectories reveal midlife-associated shifts centered around ~10 years of age, including a subset of miRNAs that show little to no change in expression during early adulthood but undergo pronounced regulatory transitions after this inflection point. These findings suggest that post-transcriptional regulatory systems undergo coordinated reorganization during midlife, paralleling the epigenetic and transcriptional

transitions identified in earlier chapters. These miRNA profiles are integrated with transcriptomic data from Chapter 2 to identify candidate miRNA-mRNA interactions that may contribute to age-related regulatory remodeling. I led this research, with co-authorship from Sam Peterson, Steven G. Kohama, and Kirstin N. Sterner.

Chapter 5 synthesizes the results of the previous chapters (2-4) and discusses broader implications and plans for future research.

Bridge to Chapter II

The introduction outlined key gaps in our understanding of hippocampal aging in long-lived primates, particularly regarding how gene regulatory systems change across the full adult lifespan and whether molecular aging follows simple linear trajectories. Addressing these gaps requires datasets with broad lifespan representation and analytical approaches capable of capturing both monotonic and nonlinear patterns of change. In Chapter 2, I leverage RNA-seq data generated from banked hippocampal samples collected from adult rhesus macaques spanning the full adult lifespan – collected through the NIH/NIA-funded Aging Primate Resource at the Oregon National Primate Research Center – to characterize transcriptomic aging across the adult lifespan. I applied analytical approaches capable of detecting both gradual, monotonic trajectories and nonlinear patterns that may reflect distinct phases of molecular aging. The findings reveal that hippocampal transcriptomic aging is not simply a uniform process of gradual decline but also involves midlife transitions that may shape vulnerability to cognitive decline and neurodegenerative disease later in life. Together, the findings of this chapter establish the transcriptomic foundation for the multi-omic framework developed in subsequent chapters.

CHAPTER II

PATTERNS OF TRANSCRIPTOMIC AGING IN THE HIPPOCAMPUS OF RHESUS MACAQUES HIGHLIGHT MIDLIFE TRANSITIONS

From Anderson, T.J., Watowich, M.M., Chiou, K.L., Goldman, E.A., Peterson, S., Anderson, J.A., Snyder-Mackler, N., Carbone, L., Kohama, S.G., and Sterner, K.N. (2025). Patterns of transcriptomic aging in the hippocampus of rhesus macaques highlight midlife transitions. GeroScience. https://doi.org/10.1007/s11357-025-01834-z.

2.1 Introduction

Aging is a complex, multifaceted process which encompasses numerous molecular and cellular changes that take place over an organism's lifespan (Aunan et al. 2016). These changes manifest at various levels, from organismal to tissue-specific, and their pace and severity can differ significantly across species. For example, some organisms exhibit negligible senescence, maintaining function and avoiding age-associated decline for much of their lives (Raz et al. 2010; O. R. Jones et al. 2014; Shefferson et al. 2017; Cohen 2018). At the tissue level, aging affects organs differently, with some tissues undergoing rapid degeneration while others maintain relative stability. In humans, brain aging is particularly consequential because it is often associated with cognitive decline driven by dysfunction in cortical and subcortical regions, although there is clear variation in the pace of brain aging between individuals. This variation is influenced by genetic, environmental, and lifestyle factors, underscoring the complexity of the aging process (Glisky 2007; Finch and Austad 2012).

While considerable research has been dedicated to uncovering the molecular mechanisms that shape brain aging (Schubert et al. 2019), much of our current understanding comes from studies that contrast young and old individuals (Fraser et al. 2005; Zhang et al. 2021). While these studies are invaluable, they have a limited ability to characterize nonlinear changes occurring over the lifespan and may thus miss critical midlife changes that shape the trajectory of aging later in life. Indeed, a growing body of research suggests that midlife is an important period for brain health outcomes in humans (e.g., cognitive decline & neurodegenerative disease) (Coleman et al. 1990; Finch 1991; Mann 1997; Debette et al. 2011; Tolppanen et al. 2012; Hatton et al. 2018; Schubert et al. 2019). For example, the association between vascular risk factors (e.g., elevated blood pressure & impaired glucose regulation) and development of Alzheimer's disease in humans shows a stronger relationship when measured at mid-life compared to when measured at late-life suggesting a need to focus interventions at this time point (Tolppanen et al. 2012).

The hippocampus is heavily involved in learning and memory; as such, it has a central role in functional aging and represents an important comparison for primate brain aging research (Bartsch and Wulff 2015; Bettio et al. 2017). Normal hippocampal aging is characterized by neurobiological alterations including increased oxidative stress and neuroinflammation, altered gene expression, reduced neurogenesis, and reduced synaptic plasticity (Bettio et al. 2017). Indeed, the hippocampus is distinguished by its enhanced neuroplasticity compared to other brain regions, which makes it more susceptible to age-related damage (Bartsch and Wulff 2015). The hippocampus is also the first brain region to suffer blood-brain barrier breakdown over the course of aging (Montagne et al. 2015). Despite its vulnerability to age-related changes, there is considerable interindividual variability in the timing and extent of decline, with the origins of

this variability yet to be fully understood (Bettio et al. 2017). Elucidating the molecular mechanisms underlying age-related changes in the hippocampus is necessary to understand drivers of cognitive decline and neurodegeneration.

Research in humans faces limitations due to the difficulty of studying neurobiological processes directly in the brain over time, particularly at the molecular level. Rhesus macaques (*Macaca mulatta*) are nonhuman primates with similar life history and conserved patterns of brain aging to humans and provide a robust translational model for understanding age-related changes in the brain (Gallagher and Rapp 1997; Herndon et al. 1997; Moss et al. 1999; Ingram et al. 2001; T. L. Moore et al. 2003; Roth et al. 2004; Chiou et al. 2020). The rhesus macaque model allows for more controlled studies that better account for factors like diet, environment and veterinary care to uncover mechanisms that may otherwise be inaccessible in human research. Using biobanked samples from a cross-section of rhesus macaques, we characterized patterns of gene expression in the rhesus hippocampus across the lifespan, which allowed us to identify midlife transitions in molecular brain aging that may relate to later brain aging.

2.2 Methods

2.2.1 Samples

We leveraged the tissue archive of the NIH/NIA-funded Aging Primate Resource at the Oregon National Primate Research Center (ONPRC) and generated RNAseq data from 96 banked hippocampus samples from rhesus macaques of different ages (3-35 years; females = 59 and males = 37) (**Table S2.1** and **Fig. S2.1**). All animals included in this study were derived from the tissue distribution program at the ONPRC, which followed the recommendations of the 2013 Edition of the American Veterinary Medical Association Guidelines for the Euthanasia of

Animals. Tissues were collected opportunistically from veterinary culls. Brains were flushed with saline, and the right hippocampus was isolated out of the medial temporal lobe, divided into thirds in the coronal plane, frozen in liquid nitrogen and archived at -80 C.

Samples used for this study were collected and banked over the course of 24 years (from 1996 to 2020). For all 96 samples, the main body of the hippocampus was pulverized in its entirety (i.e., all regions of the hippocampus: CA regions, dentate etc., are represented in the sample). Importantly, all samples included in this study showed no evidence of CNS lesions nor aberrant behaviors suggestive of brain disorders.

2.2.2 RNA extraction, library preparation and sequencing

A combined DNA/RNA extraction protocol (Qiagen DNA/RNA Allprep kit) was used to isolate genomic DNA and total RNA from these samples by the ONPRC Primate Genetics Core. Extractions were done in groups of 24 over the course of 1 day with sample order randomized to ensure not all ages/sexes were grouped together. RNAseq libraries were generated with the TruSeq Stranded Poly(A)+ protocol. Quality control was performed on all libraries by Tapestation and qPCR. Libraries were split across 3 lanes of a NovaSeq XP 200 Cycle S4 Flow Cell and sequenced with 100 bp paired-end reads at the OHSU Massively Parallel Sequencing Shared Resource core. Samples had a relatively consistent yield with an average of approximately 100 million reads per sample. All raw sequence data utilized in this analysis are publicly available (Accession: PRJNA1164722).

2.2.3 Data preprocessing

Data quality control checks of the RNAseq data were performed using FastQC (Andrews 2010). Adapter and poor-quality base trimming was performed using Trimmomatic (Bolger et al. 2014) in paired-end mode followed by an additional round of FastQC. Reads were then mapped to the rhesus macaque reference genome Mmul_10 (Accession: GCA_003339765.3) using *STAR* (Dobin et al. 2013) two-pass mode (average mapping rate: ~79%). We then used the featureCounts program (Liao et al. 2014) for quantification of raw counts of mRNA regions and the generation of a counts matrix. Data preprocessing was done using the University of Oregon's high performance computing cluster, Talapas. All subsequent data and statistical analyses were performed in R version 4.1.2. All code used in the analyses is available at (https://github.com/tannerndrsn4/Hippocampus-RNAseq-Nonlinear-Modeling)

2.2.4 Read count normalization

Read count normalization and filtering was done using the R packages edgeR (Robinson et al. 2010) and limma (Ritchie et al. 2015). The initial filtering step retained genes with sufficient expression levels across samples to ensure robust analysis. Specifically, genes were included if they had a log-transformed Counts Per Million (CPM) value above 4 in at least 15 samples. The voom function from the limma package transformed the filtered data into log2-counts per million (logCPM) values. Filtering and normalization parameters resulted in a final data set of 9,563 genes and 96 individuals for subsequent statistical analysis.

2.2.5 Differential expression analysis

A growing body of work indicates that molecular aging may follow nonlinear trajectories (Rangaraju et al. 2015; Timmons et al. 2019; Shen et al. 2024) however the extent to which this occurs in the aging rhesus macaque hippocampus is unknown. To characterize both linear (e.g., upregulated or downregulated across age) and nonlinear (e.g., midlife shifts) patterns of gene expression across the lifespan, we used autoregressive integrated moving average models (ARIMAs) – following a similar approach outlined in (Márquez et al. 2020; Watowich et al. 2022). Briefly, the best fitting ARIMA model, as identified by lowest Akaike Information Criterion (AIC) value, is determined for each gene as a function of age. The approach then selects all models which exhibit gene expression trajectories which are significantly different (FDR < 0.05) from random fluctuations in expression across the lifespan (Brockwell and Davis 2002). These genes are thus deemed to exhibit significant nonzero trends in expression; henceforth, they are referred to as differentially expressed genes (DEGs). See **Supplemental Methods** for more detail on the modeling framework and implementation.

Prior to differential expression analysis, 8 individuals were removed after discovering tissue contamination (See **Supplemental Methods, Fig. S2.2** and **Fig. S2.3**). We then performed a single round of residualization in which both sex and technical effects were jointly regressed out from our final dataset of 88 individuals. Using a linear model, we estimated the impact of sex and technical effects on expression values, then extracted the residuals to create a dataset adjusted for sex and technical effects. This approach ensured that subsequent analyses focused on age-related variation in gene expression, independent of these technical and biological confounders (See **Supplemental Methods** and **Fig. S2.4** for more detail). Differential expression analysis was performed using ARIMA models to identify DEGs and hierarchical clustering

analysis was used to group DEGs into gene clusters which share similar expression trajectories. To estimate the age at which gene expression trajectories shifted in nonlinear clusters, we fit LOESS curves to average expression values and identified the inflection point as the age with the lowest or highest predicted expression along the smoothed curve.

2.2.6 Transcriptional dysregulation during adulthood

We tested for greater variance in gene expression for older adults compared to younger adults by splitting our dataset into two age categories: 10-20 years (22 females and 13 males) and >20 years (20 females and 9 males). We focused on mid-adulthood as gene expression mechanisms become more established post-development, making adulthood a more suitable period for distinguishing age-related dysregulation from developmental changes (Somel et al. 2010).

For all 9,563 genes included in the differential expression analysis, we compared variance in expression between the age cohorts by performing F-tests on each gene while also regressing out the effects of sex and controlling for mean expression for each respective gene. To control for mean expression, we calculated mean expression for each gene in both age groups and applied a similarity threshold (0.5) to include only genes with comparable expression levels between groups (9,159 genes). This approach allowed us to analyze all genes initially, while focusing variance testing specifically on genes with similar mean expression across age groups. We then performed multiple hypothesis testing correction on the output using the R package qvalue (Storey et al. 2023) retaining only genes which passed our significance threshold (FDR < 0.05).

In addition, we tested whether the mean variance in gene expression differed significantly between age groups by performing a paired t-test on log-transformed variances across all genes. Variance values for each gene were log-transformed to stabilize variance estimates, and the mean variance was calculated for each age group to summarize overall differences in variance magnitude.

2.2.7 Gene ontology enrichment analysis

We tested for gene set enrichment on our DEGs as identified by the ARIMA approaches as well as genes determined to exhibit significant differences in variance of gene expression between age groups using an R interface for enrichR (E. Y. Chen et al. 2013; Kuleshov et al. 2016; Xie et al. 2021; Jawaid 2023). Fisher's exact test was used to determine enrichment status followed by the Benjamini-Hochberg method for multiple hypothesis test correction. For each gene set enrichment analysis, we profiled significantly enriched (FDR < 0.05) GO Biological Processes as well as KEGG pathways.

2.2.8 Transcription factor binding site enrichment analysis

We tested for transcription factor (TF) motif enrichment using ChIP-X Enrichment Analysis 3 (ChEA3) (Keenan et al. 2019). Briefly, ChIP-X Enrichment Analysis 3 (ChEA3) identifies transcription factors (TFs) likely to regulate a specified gene set by integrating multiple sources of evidence, including ChIP-seq datasets, co-expression networks, TF-target gene databases, and literature text mining. For each input gene set, ChEA3 ranks TFs based on their association across these sources, providing a comprehensive enrichment score that highlights the most relevant regulatory TFs. Genes for each expression cluster were submitted as independent

queries to provide enrichment results for each respective expression cluster. For this analysis, we chose to focus on the top 10 enriched TF for each respective query.

2.2.9 Cell-type deconvolution analysis

As cell composition can affect gene expression profiles observed in bulk RNAseq data, we characterized cell-type proportions via cell-type deconvolution using CIBERSORTx (Newman et al. 2019). This program requires the input of a signature matrix derived from rhesus macaque hippocampus single-cell RNAseq coupled with our bulk hippocampus RNAseq data to estimate cell-type proportions. To generate our signature matrix, we used data from the most recent rhesus macaque single-cell RNA-seq brain atlas (Chiou et al. 2023). We retained all cells from the hippocampus and generated average gene expression profiles for each cell type – denoted at the "cell class" level in the Chiou et al., (2023) dataset – in the dataset using the AverageExpression function in Seurat (Satija et al. 2015; Butler et al. 2018; Stuart et al. 2019; Hao et al. 2021, 2024). We then used the resulting matrix as the signature matrix required by CIBERSORTx.

Deconvolution was performed on all 9,563 genes included in our previous analyses and performed using recommended criteria for RNAseq data (e.g., disabled quantile normalization & 100 permutations for significance analysis). First, we quantified whether cell-type significantly differed across age in the hippocampus of rhesus macaques using linear regression on the estimated cell proportions as a function of age for each cell type. Secondly, we used the CIBERSORTx output to control for cell-type change across age in an additional ARIMA approach which aimed to characterize gene expression changes across age independent of cell-type or sex effects using a similar approach as described above.

2.3 Results

2.3.1 Genes differentially expressed across age fit four broad expression patterns

Our preliminary data exploration highlighted a potential batch effect in the data along with the presence of outliers (See **Supplemental Methods**). Briefly, we removed 8 individuals (**Table S2.1**) from the dataset (6 individuals with evidence of choroid plexus tissue contamination and 2 extreme outliers) and corrected for technical effects, resulting in a final dataset of 88 individuals (55 female & 33 male) (**Fig. 2.1A**). We performed PCA analysis on this dataset (**Fig. 2.1B** and **2.1C**) and found PC2 to be significantly associated with age (β = 0.6057, 95% CI [0.3603, 0.8511], p = 4.30e-06) (**Fig. 2.1D**).

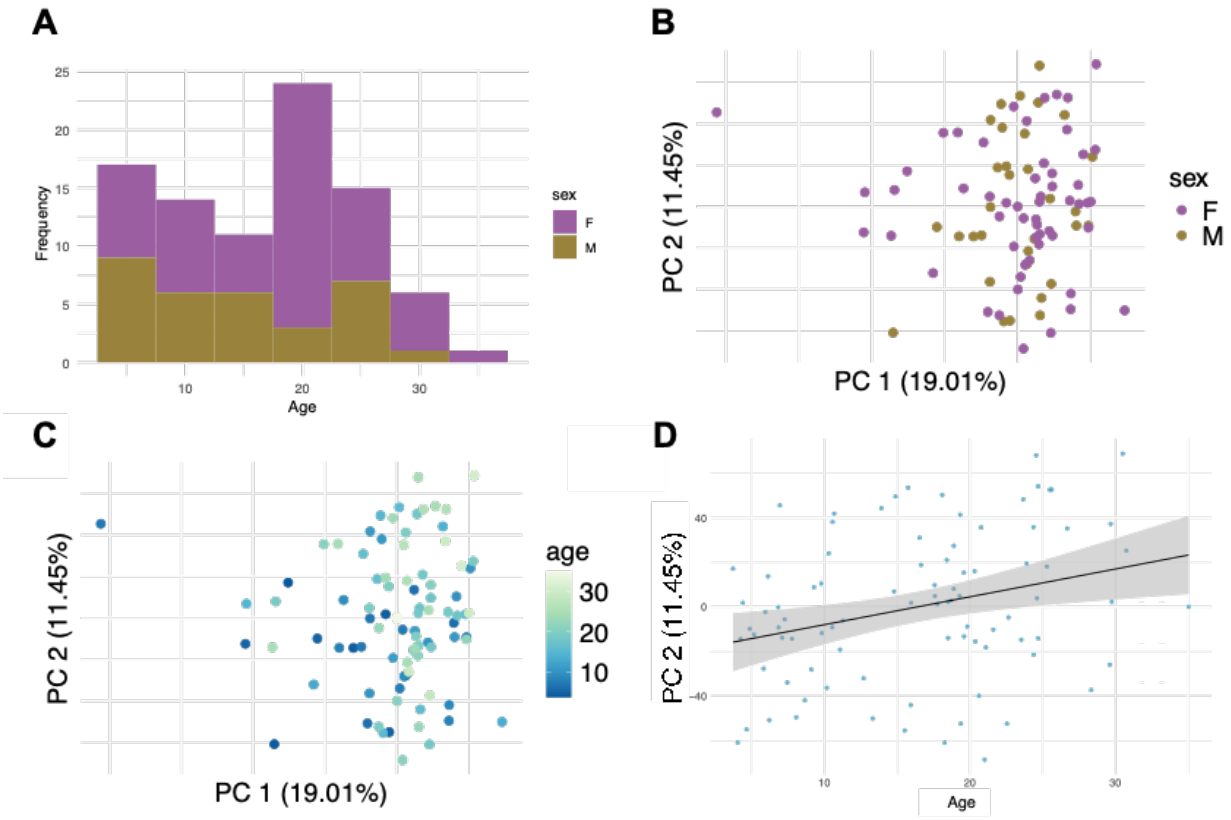
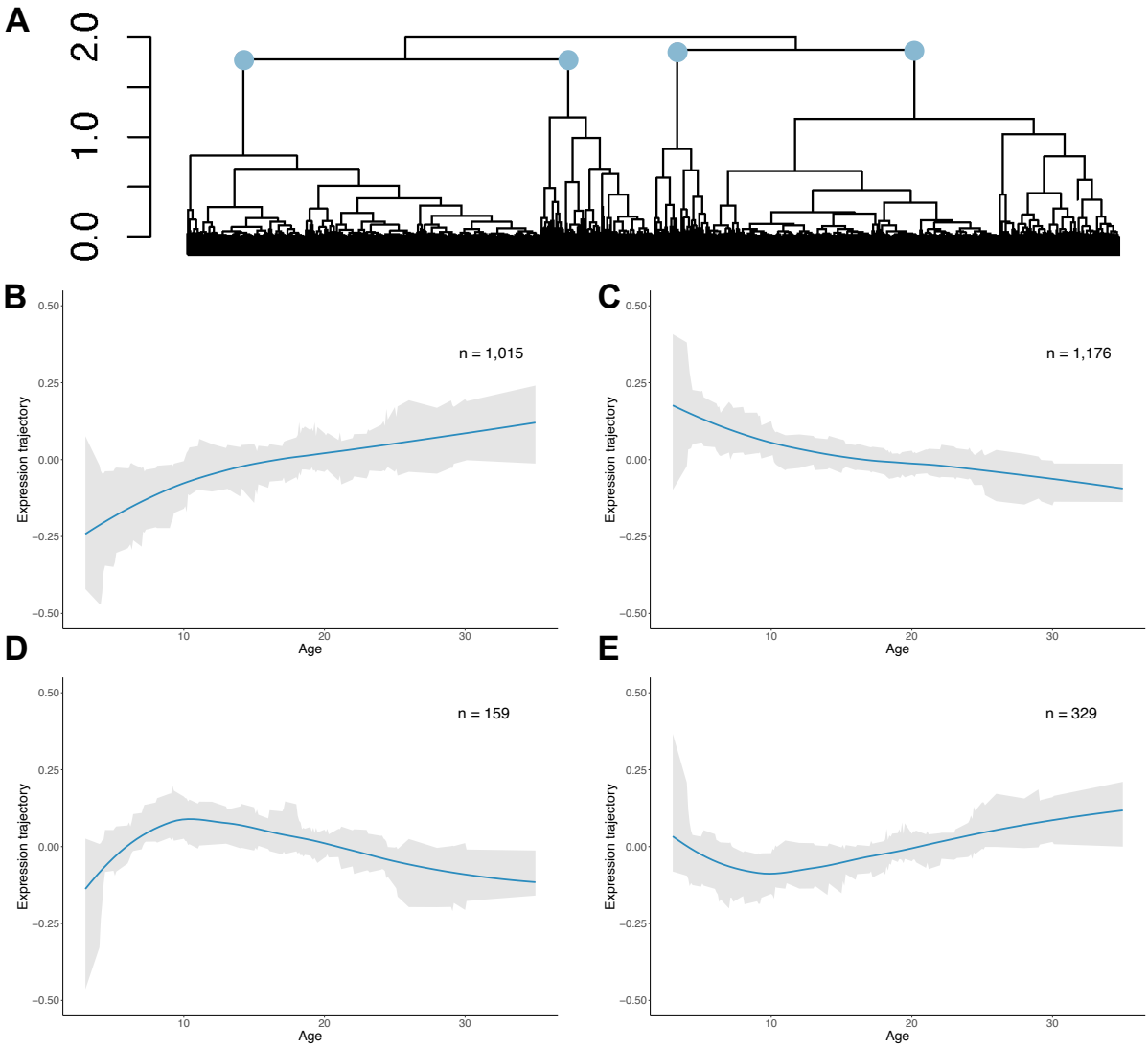


Fig. 2.1 Sample distribution and PCA analysis for 88 rhesus macaque hippocampus samples. A) Distribution of ages. Samples include representation across the lifespan and both males and females. Each bar represents an age range of 5 years. B) PCA analysis of all 88 individuals included in differential expression analysis as denoted by sex, C) and by age. D) PC2 was found to be significantly associated with age (β = 0.6057, 95% CI [0.3603, 0.8511], p = 4.30e-06)

We identified 2,679 (28%) age-associated differentially expressed genes (DEGs) (FDR < 0.05) and identified 4 broad clusters in temporal gene expression using hierarchical clustering (**Fig. 2.2A**). Two clusters (Cluster 1 [1,015 genes; 37.9%] and 2 [1,176 genes; 43.9%]) demonstrated linear trends in gene expression (**Fig. 2.2B** and **2.2C** respectively) while the other two (Cluster 3 [159 genes; 5.9%] and Cluster 4 [329 genes; 12.3%]) demonstrated nonlinear trends (**Fig. 2.2D** and **2.2E** respectively).

Fig. 2.2 (next page) Hierarchical clustering and expression trajectories for DEGs that fit 4 broad expression patterns. A) Cluster Dendrogram of all significantly differentially expressed genes as identified by ARIMA modeling when controlling for sex. Clusters utilized in analysis are denoted by blue nodes. B-E) Mean predicted expression counts for all genes in each cluster, as determined by ARIMA models. The blue line (LOESS-smoothed curve) represents the average trajectory, with a shaded ribbon highlighting ±1 standard deviation around the mean. Number of genes in each cluster is denoted by n



Cluster 1 contained 1,015 genes (37.9% of DEGs) upregulated across age (**Fig. 2.2B**) and GO term analysis found enrichment for genes largely implicated in positive regulation of transcription and DNA-templated transcription (GO:0045893) and sphingolipid metabolic process (GO:0006665) (**Table S2.2**). Enriched KEGG pathways contained 15 significant pathways after FDR correction, including CNS-related terms (e.g., Notch signaling pathway, Sphingolipid metabolism and Neurotrophin signaling pathway and cellular pathways, Lysosome, Adherens junction, Autophagy) (**Fig. 2.3A** and **Table S2.2**). Inflammatory pathways (e.g., amyloid-beta formation [GO:0034205]) also show enrichment although they are not among the

top enriched GO terms and KEGG pathways (**Table S2.2**). Enriched TFs included zinc fingers (e.g., ZKSCAN8 and ZNF507) as well as MYRF, a myelin regulatory factor, and Bromodomain and PHD Finger Containing Protein 1, a transcriptional regulator implicated in neuron remodeling that has also been implicated in Alzheimer's disease progression (Jordan-Sciutto et al. 2001) (**Table S2.3**).

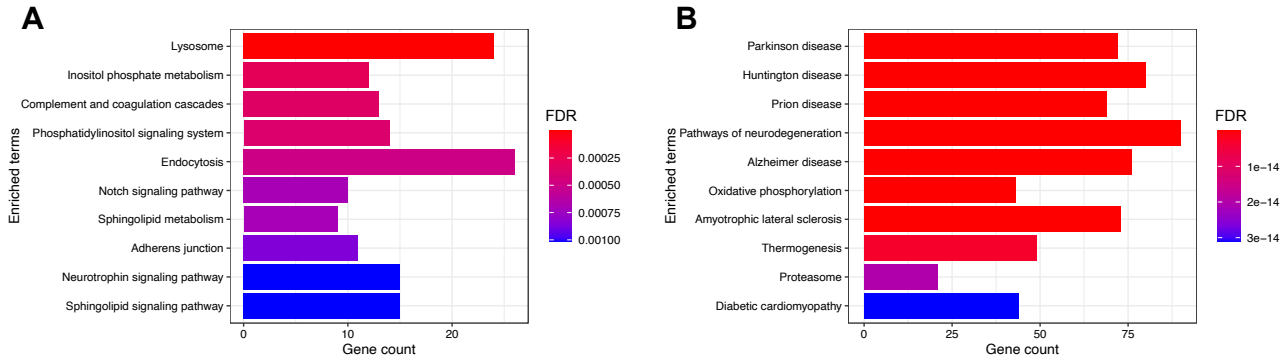


Fig. 2.3 KEGG pathway enrichment analysis for genes exhibiting linear expression trajectories. A) Top 10 enriched KEGG pathways after FDR correction (FDR < 0.05) for genes upregulated across age, and B) genes downregulated across age using EnrichR

Cluster 2 contained 1,176 genes (43.9% of DEGs) downregulated across age (**Fig. 2.2C**). GO analysis found enrichment for 136 pathways after FDR, including GO term enrichment for aerobic electron transport chain (GO:0019646), mitochondrial ATP synthesis coupled electron transport (GO:0042775) and mitochondrial respiratory chain complex I assembly (GO:0032981) and several neurobiological categories (**Table S2.2**). Enriched KEGG pathways for these genes listed several neuro-related diseases including Parkinson disease, Alzheimer disease and pathways of neurodegeneration (**Fig. 2.3B** and **Table S2.2**). Enriched TFs included zinc fingers (e.g., DPF1, ZNF428, SCRT1, GTF3A, and ZNF511) as well as Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, an enzyme that has been implicated in the pathogenesis of Alzheimer's disease and many cancers (Driver et al. 2015) (**Table S2.3**).

Cluster 3 contained 159 genes (5.9% of DEGs) with nonlinear trends in expression; specifically, these genes were found to increase in expression before decreasing later in life with an inflection point centered around ~10 years, as estimated from the LOESS-smoothed expression curve (9.5 years) (**Fig. 2.2D**). GO analysis did not identify any significantly enriched GO terms for the genes in this cluster after p-value correction and KEGG pathway enrichment identified only 1 significantly enriched pathway: amphetamine addiction (**Table S2.2**). The top enriched TF for the genes in Cluster 3 was zinc finger ANKZF1 (**Table S2.3**). Indeed, zinc fingers were highly enriched for the genes in this cluster as several others appeared in the top 10 enriched TFs (e.g., ZNF692, RBCK1, ZNF540, CXXC1, GLI4 and ZBED5) (**Table S2.3**). In addition, Neuronal Differentiation 6, a basic helix-loop-helix transcription factor involved in the development and differentiation of the nervous system (Khan et al. 2017) was also enriched.

Cluster 4 contained 329 genes (12.3% of DEGs) which also demonstrated nonlinear trends in gene expression; specifically, these genes decreased in expression before increasing again later in life with a similar inflection point centered around ~10 years, as estimated from the LOESS-smoothed expression curve (9.2 years) (**Fig. 2.2E**) – exhibiting an inverse pattern to Cluster 3. GO analysis showed enrichment for genes related to histone modifications (GO:0070932) and transcriptional regulation (GO:1903507) (**Table S2.2**). There were no KEGG terms significantly enriched for the genes in this cluster after p-value correction (**Table S2.2**). The top enriched TF for the genes in this cluster was Homeobox protein Nkx-6.2, a TF implicated in myelination regulation and associated with spastic ataxia in humans, a disease characterized by a general hypomyelination of the central nervous system which can lead to a number of neurological complications or death (Chelban et al. 1993; Shurrab et al. 2023) (**Table S2.3**). Other enriched TFs included zinc fingers (e.g., ZNF853 and ZBTB47), oligodendrocyte

transcription factor OLIG1, MYRF, and box genes SOX10 and SOX8 which are implicated in brain development and function (Nelms and Labosky 2010).

We also aimed to determine whether these identified expression patterns were maintained when looking within sex. To accomplish this, we divided our 88 individuals into males (N = 33) and females (N = 55) and reran our differential expression analyses (**Fig. S2.5**). With these models, we identified 2,068 DEGs (~80% overlap with original approach) in females (**Table S2.4** and **Fig. S2.6**) and 1,742 DEGs (~65% overlap with original approach) in males (**Table S2.5** and **Fig. S2.7**) (FDR < 0.05). Hierarchical clustering analysis suggested that differentially expressed genes for both females and males grouped into 4 broad expression patterns that largely mirrored the patterns of the original approach (see **Supplemental Results** and **Discussion**).

2.3.2 Older adults exhibit increased variance in expression compared to younger adults

We identified 24 genes (FDR < 0.05) which exhibited greater variance in individuals aged ~10-20 years (n = 35) compared to 291 genes in individuals >20 years of age (n=29). While pathway analysis did not identify any significantly enriched GO terms in the first group of genes, we identified 39 significantly enriched GO terms for the 291 genes in the second group and a number of them are implicated in brain-related processes (e.g., transport across blood-brain barrier [GO:0150104] & positive regulation of neurogenesis [GO:0050769]) (**Table S2.6**). In addition, mean variance across genes was found to be significantly higher in individuals >20 years of age (0.0645) compared to individuals aged 10-20 years (0.0528) (t = -32.89, df = 9550, p = 2.2e-16, mean difference = -0.170). Collectively, these findings may suggest a disruption in gene regulatory control of neuronal processes associated with increased age.

2.3.3 Deconvolution analysis identifies known age-related changes in oligodendrocytes and glutamatergic neurons

To infer how cell types change across age in the rhesus hippocampus, we employed cell-type deconvolution analysis using CIBERSORTx. This analysis revealed that the majority of our 12 reported cell types, which include neurons and glial cells (as denoted in the signature matrix based on the hippocampal dataset of Chiou et al., (2023)), do not appear to significantly change in proportion across age (**Table S2.7**).

However, two cell types appear to differ in abundance across the lifespan: oligodendrocytes significantly increase in proportion across age ($\beta = 0.00099$, 95% CI [0.00012, 0.00186], $p = 0.026$) while glutamatergic neurons significantly decrease in proportion across age ($\beta = -0.0017$, 95% CI [−0.0029, −0.0006], $p = 0.003$) (**Fig. 2.4A** and **Fig. 2.4B** respectively). The observed increase in oligodendrocytes is consistent with previous observations in rhesus macaques (Luebke et al. 2010; Chiou et al. 2022) although it is not unique to the hippocampus. The observed decrease in glutamatergic neurons is consistent with decreases in glutamate concentration observed with age in human brains (Kaiser et al. 2005; Roalf et al. 2020; Gasiorowska et al. 2021). We did not observe a significant increase in microglia proportions associated across age as we might expect given increased activation and density of microglia with age in human brains (Edler et al. 2021), but see (Alan Peters et al. 2008; Kanaan et al. 2010; Chiou et al. 2022) and **Supplemental Results** and **Discussion**.

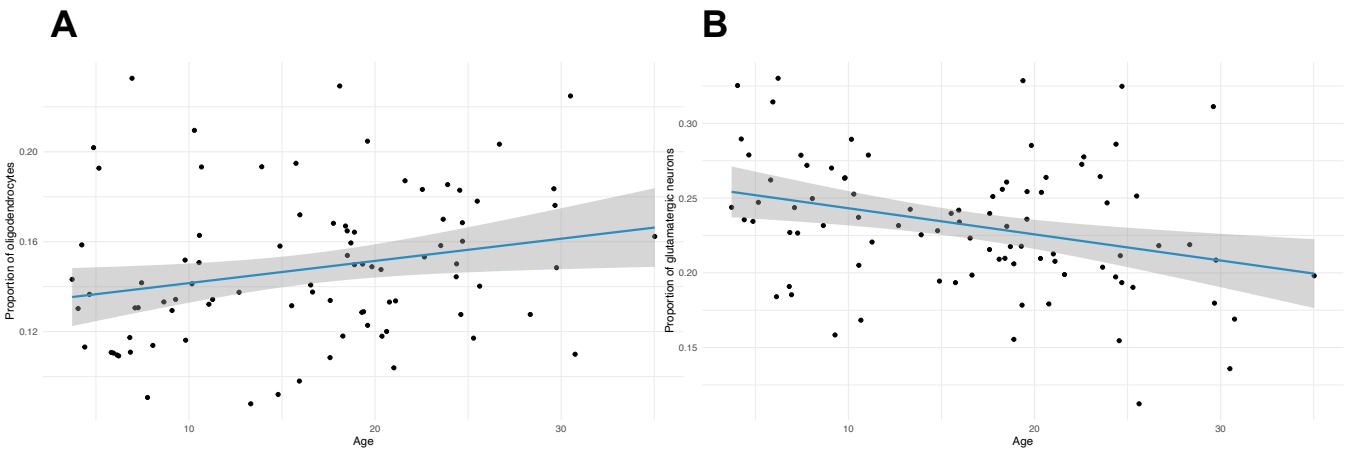


Fig. 2.4 Proportion of significant cell type change across age. A) Linear regression of oligodendrocyte (β = 0.00099, 95% CI [0.00012, 0.00186], p = 0.026) and B) glutamatergic neuron β = -0.0017, 95% CI [−0.0029, −0.0006], p = 0.003) cell type proportion change across age

To examine gene expression changes independent of changes in cell-type proportions, we performed an additional ARIMA analysis controlling for the effects of estimated cell-type proportions. This analysis identified 2,177 age-associated DEGs (FDR < 0.05). Hierarchical clustering analysis revealed that these genes largely fit 4 broad expression patterns (**Fig. S2.8**). The expression patterns largely mirrored the patterns of the original (non-cell-type corrected) approaches with the first two clusters exhibiting largely linear (i.e., monotonic) trends (Cluster 1 = 1,026 genes; Cluster 2 = 845 genes) while the other two clusters exhibited nonlinear trends (Cluster 3 = 188 genes; Cluster 4 = 118 genes) (**Fig. S2.9**). Approximately ~71% of these genes overlapped with the genes identified by our original approach. Most of the overlap was found between the linear clusters (Cluster 1 overlap = 97.1%; Cluster 2 overlap = 86.9%) with no overlap identified in the nonlinear clusters (Cluster 3 overlap = 0%; Cluster 4 overlap = 0%). This approach also showed a moderate similarity in effect size to the original model (r = 0.32) as determined by calculating the correlation between the two sets of coefficients for the common genes, focusing on the diagonal elements to compute an average correlation value that summarizes the similarity between the two models. This may suggest that cross-sectional

differences in cell type composition are impacting fluctuations in gene expression between time points, despite strong overlap in gene expression patterns at large – particularly in the linear clusters. However, this interpretation should be approached with caution, as it is based on the signature matrix used (see **Supplemental Discussion**).

2.4 Discussion

We characterized age-associated gene expression changes in the hippocampus of rhesus macaques across the lifespan and identified a set of age-associated genes that exhibit nonlinear patterns of expression–findings that may provide novel insight into the molecular mechanisms associated with brain aging during midlife. Specifically, these nonlinear patterns revealed expression minima (or maxima) at ~10 years of age (approximately 30 years in humans) (**Fig. 2.2D** and **2.2E**), suggesting this as a key transition point between molecular development and decline of the hippocampus. We refer to this inflection point as ~10 years throughout the main text to provide a clear and interpretable age division, while acknowledging that it does not represent a precise biological transition common to all individuals. Importantly, this finding supports growing interest in characterizing more dynamic and nonlinear patterns of aging at the molecular level. For example, a recent multi-omics profiling study of human biological samples (e.g., blood, stool, skin swab, oral swab and nasal swab samples) found that many molecular patterns of aging are nonlinear, with periods of significant change occurring during mid-life (Shen et al. 2024).

Studies have shown that midlife in humans is associated with a variety of biological and environmental factors that cumulatively contribute to the health of the brain during old age (Yaffe et al. 2012; Lockhart and DeCarli 2014). The physiological changes occurring during this

period (e.g., metabolic alterations), alongside lifestyle factors, can have a profound impact on the hippocampus, potentially predisposing individuals to cognitive decline and increasing their risk of neurodegenerative conditions such as Alzheimer's disease (Raz et al. 2010; Schubert et al. 2019). There are two non-mutually exclusive explanations for the shift in expression trajectories we observed in rhesus at ~10 years of age.

First, the signal reflects the completion of brain maturation in macaques. It is widely understood that human brains undergo a "rewiring" process and do not reach full maturation until the age of 25 (Gavin et al. 2009; Arain et al. 2013). It is therefore possible that the molecular shift identified in our nonlinear expression trajectories are reflective of "completed" maturation of the rhesus macaque brain. This hypothesis is supported by our enrichment results. Specifically, our TF enrichment analysis found that DEGs that shift expression at ~10 years are associated with box proteins (SOX10 and SOX8), transcription factors important for neural crest development and brain differentiation generally. Additionally, (W. Wang et al. 2022) identified *ETNPPL* as a primate-specific neural stem marker playing an important role in adult hippocampal neurogenesis. We found this gene to be differentially expressed across age specifically exhibiting nonlinear patterns of expression (Cluster 4). This further highlights a potential transition point between the developing and mature rhesus macaque hippocampus.

Second, the signal reflects changes in white matter volume associated with aging. Studies have revealed that white matter (i.e., regions containing myelinated axons) volume peaks at approximately 30 years of age in humans and declines thereafter (S. Wang and Young 2014; Cox et al. 2016; Jiskoot et al. 2018; Reas et al. 2018), with the greatest degree of decline being detected in the prefrontal cortex and anterior corpus callosum (Bartzokis et al. 2003, 2010; Bennett et al. 2009). Indeed, a decrease in white matter volume is a hallmark of general primate

brain aging (Guttmann et al. 1998; Bartzokis et al. 2010). In rhesus macaques, (Kubicki et al. 2019) identified that white matter volume peaks around ~10 years of age matching known patterns in humans. Additionally, (Dash et al. 2023) found that white matter volumes in macaques exhibit an arc-shaped trajectory with peaks occurring in middle age. These trends directly correspond to the shift observed in our nonlinear clusters and suggest that the genes in these clusters may be impacted by or drive this change in white matter volume. This may be the most likely explanation for the molecular shift observed in our nonlinear clusters as it is supported by the results of our cell-type deconvolution analysis. Specifically, the observed oligodendrocyte proportion increase associated with increased age. The primary function of oligodendrocytes in the brain is to produce and maintain myelin which ensheathes axons in order to increase speed and efficiency of axon signal conduction (Skoff and Benjamins 2014). Increased oligodendrocyte proportion associated with age is argued to be a compensatory mechanism to combat the detrimental effects of white matter loss through remyelination (Sams 2021). TF enrichment analysis also supports this hypothesis with TFs implicated in myelin regulation (MYRF) and oligodendrocyte differentiation (OLIG1 and OLIG2) associated with DEGs exhibiting nonlinear patterns of expression (DEGs in Clusters 3 & 4).

Definitions of midlife vary with some studies specifying the midlife period as between the ages of 40-65 years in humans (Infurna et al. 2023) and others more broadly referring to midlife as a period between youth and old age (Lachman 2004). There is even less clarity when defining this period across species. For example, many descriptions of midlife in macaques simply reference adulthood or middle of the lifespan (Y. Ma et al. 2023). Rhesus macaques age at roughly 2-3 times the rate of humans and with sexual maturity typically occurring between 2.5-4.5 years, full body size being reached between 5-7 years, and a median lifespan of ~27 years

(Colman et al. 2009; Lewis and Prongay 2015; Chiou et al. 2020). For the sake of this study, we broadly define midlife in rhesus macaques as referring to any age between sexual maturation (~3-6 years) and old age (~25 years) (Lewis and Prongay 2015; Simmons 2016). Our study suggests that this general timepoint (and the comparable timepoint in humans) could be an important transitionary period between normal hippocampal development and age-associated decline and thus may provide insight into the mechanisms that underpin the susceptibility and resilience of the hippocampus to age-related pathologies. Indeed, midlife represents a pivotal period in the human lifespan with regard to health outcomes, serving as a critical juncture that can significantly influence the aging trajectory of the hippocampus (C. K. Lee et al. 2000).

Similar to others, we found that the majority of differentially expressed genes exhibited linear trends in expression and are enriched for GO terms and pathways known to be upregulated (e.g., transcriptional regulation and inflammatory pathways; **Table S2.2**) or downregulated (e.g., metabolism and mitochondrial dysfunction; **Table S2.2**) with age respectively. Specifically, previous research has shown that inflammatory/immune response genes and pathways are upregulated in the hippocampus of older rodents (C. K. Lee et al. 2000; Rowe et al. 2007), rhesus macaques (Blalock et al. 2010) and humans (T. Lu et al. 2004; McGeer and McGeer 2004; Parachikova et al. 2007). This pattern may relate to increased microglial activation and dysfunction during aging (Ogura et al. 1994; Griffin et al. 2006). Importantly, exaggerated pro-inflammatory responses associated with age in humans (Rawji et al. 2016) can increase susceptibility to neurodegenerative diseases, as chronic brain inflammation is a pathological hallmark of Alzheimer's disease (Balin and Hudson 2014). Previous studies have also shown that genes involved in synaptic function, neuronal signaling and synaptic plasticity are downregulated in the hippocampus of older primates (Loerch et al. 2008). In addition, genes involved in neural

development, signaling pathways and metabolic processes, which indicate a shift in cellular metabolism and function with age, have also been shown to be downregulated with age in the hippocampus of rodents (Kadish et al. 2009).

Interestingly, KEGG pathways for neuro-related diseases (e.g., Parkinson disease & Alzheimer's disease) were also enriched in DEGs downregulated across age (**Table S2.2**), identifying a set of genes that may be particularly relevant to understanding resilience mechanisms in aging primates. Many of these genes are involved in mitochondrial function, including regulators of apoptosis (e.g., *BAX*, *HTRA2*, *TOMM40*, *MCU*, *HSD17B10*) and components of oxidative phosphorylation (e.g., *ATP5F1C*, *ATP5F1E*, *COX7B*, *COX8A*), which are critical for maintaining cellular energy balance and neuronal health. Although mitochondrial dysfunction is a hallmark of aging and a key feature of neurodegenerative disease in humans (López-Otín et al. 2023), the downregulation of these genes in aging macaques may reflect an alternative regulatory trajectory that reduces apoptotic and metabolic stress to support neuronal resilience.

While this study was not designed to test the functional consequences of age-related gene expression changes directly, our findings highlight several candidates that warrant future investigation through functional validation approaches. For example, *BAX* is a pro-apoptotic member of the BCL2 family that promotes mitochondrial outer membrane permeabilization and initiates caspase activation during apoptosis (Oltval et al. 1993; Yin et al. 1994). In humans, studies show that *BAX* expression is significantly elevated in the frontal cortices of individuals with Alzheimer's disease compared to those with normal aging, suggesting that increased *BAX* expression contributes to neuronal vulnerability (G. Lu et al. 2005). In contrast, downregulation of *BAX* in the aging macaque hippocampus, albeit a different brain region, may reflect an

adaptive shift toward lower apoptotic sensitivity, preserving neuronal viability with age. Thus, *BAX* downregulation in macaques could represent a brain-specific resilience mechanism, consistent with prior findings that macaques maintain higher expression of genes related to neurogenesis and synaptic plasticity into adulthood (Y. Zhu et al. 2018), and do not exhibit the widespread neuronal loss characteristic of Alzheimer's disease (Keuker et al. 2003). Understanding the role these genes (**Table S2.2**) play in hippocampal aging and how they are differentially regulated across species may reveal important clues to human-specific vulnerability to neurodegeneration and disease (Loerch et al. 2008).

The age-associated increase in gene expression variance was also consistent with previous studies which observed this phenomenon both in the brain and in tissues throughout the body both in humans (Viñuela et al. 2018) and rhesus macaques (Chiou et al. 2022). Age-related increases in variance of expression are thought to be explained by decline in gene regulatory control mechanisms (i.e., dysregulation) which contribute both to general aging and age-associated diseases (Yerbury et al. 2016; Nativio et al. 2018). We found that genes which exhibited increased variance in expression for individuals >20 years of age were enriched for brain-related GO terms including transport across blood-brain barrier, positive regulation of neurogenesis and synapse pruning (**Table S2.6**). These terms provide potential biological mechanisms which may be impacted by or implicated in the loss of gene regulatory control associated with age. Indeed, the hippocampus is distinct from other brain regions in that it is one of the first to suffer breakdown in the blood-brain barrier (Montagne et al. 2015). Our results suggest that increased variance in expression may relate to this breakdown, although the mechanism and direction of interaction requires further investigation.

Finally, several genes known to be integral in facilitating the anti-aging effects of calorie restriction also demonstrated age-associated patterns of differential expression in the hippocampus of rhesus macaques. Many of these transcripts follow linear trajectories in expression (e.g., *IGF1R* & *FOXO3* are upregulated across age), however two, exhibit nonlinear trajectories of expression. Specifically, *SIRT1* and *SIRT2* have more "U-shaped" expression trajectories, reaching their lowest expression levels at ~10 years of age (**Fig. S2.10**). The sirtuin genes are argued to play pivotal roles in neuroprotection and cellular senescence (Manjula et al. 2021). Both *SIRT1* and *SIRT2* encode NAD+-dependent protein deacetylases involved in the regulation of glucose metabolism, insulin signaling, and oxidative stress protection (F. Wang et al. 2007; Kitada and Koya 2013). Sirtuin 1 promotes neuronal health during aging through supporting axonal elongation, neurite outgrowth, dendritic branching, and memory formation by modulating synaptic plasticity (Herskovits and Guarente 2014). Sirtuin 2 deacetylates the transcription factor Forkhead Box Protein O3 in response to oxidative stress and calorie restriction, promoting cellular survival pathways (Keuker et al. 2003). Given their importance in anti-aging/neuroprotection (Donmez and Outeiro 2013), midlife changes in these genes and pathways warrant further study.

This study provides valuable insights into molecular changes occurring during midlife in the aging rhesus macaque hippocampus; however, several limitations of the dataset warrant consideration. First, the analysis was conducted on RNA derived from previously banked whole tissue samples. This approach provides a broad molecular overview but is inherently limited in its resolution. While we include deconvolution analyses, these types of approaches are only able to infer cellular changes and do not allow for direct observation of cell-specific changes and insights that would be possible using single-cell RNA sequencing. Second, the absence of

correlative behavioral data limits the ability to control for cognitive or functional variability that might otherwise provide additional context for the observed molecular changes. Third, the samples were collected over a 25-year period, introducing potential variability. Nonetheless, all samples included in the study were confirmed to be free of overt neuropathologies, and the post-mortem interval prior to tissue storage was minimized to ensure sample integrity. Despite these limitations, this study addresses a critical gap in understanding the molecular landscape of hippocampal aging in primates, a topic largely uncharacterized due to the scarcity of available samples. Moreover, while the study is not longitudinal, the inclusion of a large sample size with replicates of individuals at similar ages strengthens the reliability of the findings.

This study characterizes transcriptomic aging in the hippocampus of rhesus macaques, revealing critical molecular transitions that occur during midlife. We identified both linear and nonlinear patterns of gene expression that provide novel insights into the aging process, particularly during this pivotal stage of life. The significant shifts in gene expression observed in rhesus macaques at ~10 years of age (~30 years of age in humans) suggest that midlife may represent a critical juncture in hippocampal molecular aging. These nonlinear trajectories, coupled with the observed changes in oligodendrocyte proportions and white matter volume, highlight the potential role of cellular composition in influencing gene expression during this period. Furthermore, our results align with broader patterns observed in humans and other primates, underscoring the importance of midlife as a period where molecular and cellular changes may predispose the brain to age-related pathologies (López-Otín et al. 2023). Importantly, this study emphasizes the need for future research to dissect the specific roles these differentially expressed genes play in shaping aging phenotypes and age-associated diseases,

especially as nonlinear patterns of gene expression may uncover novel mechanisms driving susceptibility or resilience to neurodegenerative conditions.

Bridge to Chapter III

Chapter 2 established that hippocampal transcriptomic aging in rhesus macaques is not simply a linear process of gradual decline but also involves distinct molecular trajectories – including a midlife transition centered around ~10 years of age – that may set the stage for later-life vulnerability to cognitive decline and neurodegeneration. These transcriptional patterns raise a fundamental question: what upstream regulatory mechanisms drive and coordinate these age-associated changes in gene expression? Chapter 3 addresses this question by characterizing the epigenetic landscape of hippocampal aging through the lens of DNA methylation (DNAm), a key mechanism of gene regulatory control. By profiling DNAm across the same adult lifespan-spanning cohort of rhesus macaques, Chapter 3 asks how age-associated changes in the methylome manifest, and whether regions of epigenetic remodeling coincide with genes and regulatory elements implicated in age-associated transcriptional change. Together, these two chapters form the epigenomic and transcriptomic backbone of the multi-omic framework developed across this dissertation, building toward an integrated understanding of how molecular regulatory systems change across the primate lifespan.

CHAPTER III

DNA METHYLATION REMODELING ACROSS THE ADULT LIFESPAN IN THE RHESUS MACAQUE HIPPOCAMPUS

3.1 Introduction

Aging is a complex biological process characterized by progressive physiological decline and increased susceptibility to chronic disease (J. Guo et al. 2022). The pace of aging varies across species, individuals, and tissues, and is shaped by molecular regulatory processes that influence cellular function across the lifespan (Raz et al. 2010; Goldman and Sterner 2023; Fraga 2009). Recent frameworks have organized these processes into interconnected hallmarks, including genomic instability, mitochondrial dysfunction, altered intercellular communication, and epigenetic alterations (López-Otín et al. 2023). Among these, disruptions to gene regulation, via modifications to the epigenome, are increasingly recognized as central contributors to aging phenotypes (Aunan et al. 2016; López-Otín et al. 2023).

Inter-individual variation in organ-specific aging is conspicuous in the brain, where some individuals maintain cognitive function into advanced age while others develop neurodegenerative diseases such as Alzheimer's disease (AD) (Langa 2018; Patel et al. 2022; Disouky et al. 2026). In the human brain, age-associated epigenetic alterations, specifically DNA methylation (DNAm) changes, are highly region-specific, with pronounced remodeling in cortical and hippocampal regions (Covic et al. 2010; Pellegrini et al. 2021). These age-associated differential methylation patterns frequently overlap those observed in neurodegenerative disease, consistent with accelerated epigenetic aging observed in individuals with AD (Pellegrini et al. 2021). DNAm alterations in the hippocampus have also been associated with pathological

features such as amyloid deposition and neurofibrillary tangles (Chouliaras et al. 2013), and may reflect increasing epigenetic instability that contributes to loss of neuronal identity and homeostasis (Jeong et al. 2025). Together, these findings suggest that differences in molecular regulatory stability, including epigenetic regulation, may contribute to variation in susceptibility to age-associated cognitive decline (Cui and Xu 2018; Harman and Martín 2020; Marzola et al. 2023). Despite these insights, most work has focused on disease states or late-life comparisons, and comprehensive understanding of how epigenetic regulation changes across the lifespan in healthy primate brains remains limited.

Among brain regions, the hippocampus is especially sensitive to age-associated change (Bartsch and Wulff 2015). As a structure central to learning, memory, and neuroplasticity, it exhibits pronounced aging-associated alterations, including synaptic dysfunction, altered neurogenesis, inflammatory signaling, and increased vulnerability to neurodegenerative disease (Harman and Martín 2020; Bettio et al. 2017).

DNAm provides a key mechanism for interrogating age-associated changes in gene regulation. Across tissues and species, DNAm exhibits predictable shifts with age (Horvath 2013; Teschendorff et al. 2013; Levine et al. 2018; T. Zhu et al. 2018; López-Gil et al. 2023). These changes include methylation gains at CpG-rich promoters and Polycomb-repressed regions, alongside methylation loss in CpG-poor intronic and intergenic regions enriched for enhancer activity (Rakyan et al. 2010; Teschendorff et al. 2010; Day et al. 2013; Fernández et al. 2015; Pérez et al. 2018; Voisin et al. 2021), suggesting coordinated remodeling of regulatory elements. In parallel, the methylome becomes increasingly variable with age, as tightly regulated CpG sites exhibit greater inter-individual dispersion – a phenomenon described as epigenetic drift or loss of regulatory fidelity (Hannum et al. 2013; Field et al. 2018; Martin-Herranz et al.

2019; Seale et al. 2022). The majority of these sites increase in variance across the lifespan, though a minority converge toward fully methylated or unmethylated states (Fernández et al. 2015; Hannum et al. 2013; Oh et al. 2016; Phipson and Oshlack 2014; Vershinina et al. 2021). Together, these patterns indicate that aging reshapes the methylome through both structured regulatory changes and increasing stochastic variation.

Most studies of brain DNAm aging have relied on comparisons between young and old individuals or have assumed predominantly linear trajectories (Fraser et al. 2005; Horvath et al. 2012; Langie et al. 2017; Zhang et al. 2021). Increasing evidence suggests that age-associated DNAm changes encompass multiple dimensions – including shifts in mean methylation, increases in variability, and nonlinear trajectories across the lifespan (Unnikrishnan et al. 2018; Lapborisuth et al. 2022; Seale et al. 2022; Olecka et al. 2024; Shen et al. 2024). Such nonlinear dynamics may reflect transition periods that influence later-life aging trajectories in longer lived species, yet these dynamics remain poorly characterized in the hippocampus.

Rhesus macaques (*Macaca mulatta*) have a similar life history to humans and provide a translationally relevant model for human brain aging (Gallagher and Rapp 1997; Herndon et al. 1997; Moss et al. 1999; Ingram et al. 2001; T. L. Moore et al. 2003; Roth et al. 2004; Chiou et al. 2020). Here, we characterize age-associated DNAm patterns across the adult lifespan in the rhesus macaque hippocampus using reduced representation bisulfite sequencing (RRBS). By integrating single-CpG and region-level analyses, we quantify methylation gains, losses, variance changes and model both monotonic (i.e., linear) and nonlinear shifts. Leveraging matched hippocampal RNAseq data from the same individuals (T. J. Anderson et al. 2025), we evaluate whether age-associated methylation regions intersect genic and distal intergenic features with potential *cis*-regulatory relevance. Together, this multi-scale framework provides insight

into how epigenetic remodeling may shape brain aging trajectories in a region central to learning, memory, and vulnerability to neurodegenerative disease.

3.2 Methods

3.2.1 Sample Information

This study leveraged banked hippocampal samples sourced from 86 rhesus macaques (n=56 female, n=30 male) ranging in age from ~3 to 35 years (**Fig. 3.1** and **Table S3.1**). All individuals in this cohort were reproductively mature, and many had reached advanced ages for the species, with individuals up to 35 years old – well beyond the approximate captive lifespan of ~25 years (Chiou et al. 2020) – enabling characterization of molecular aging across the adult lifespan.

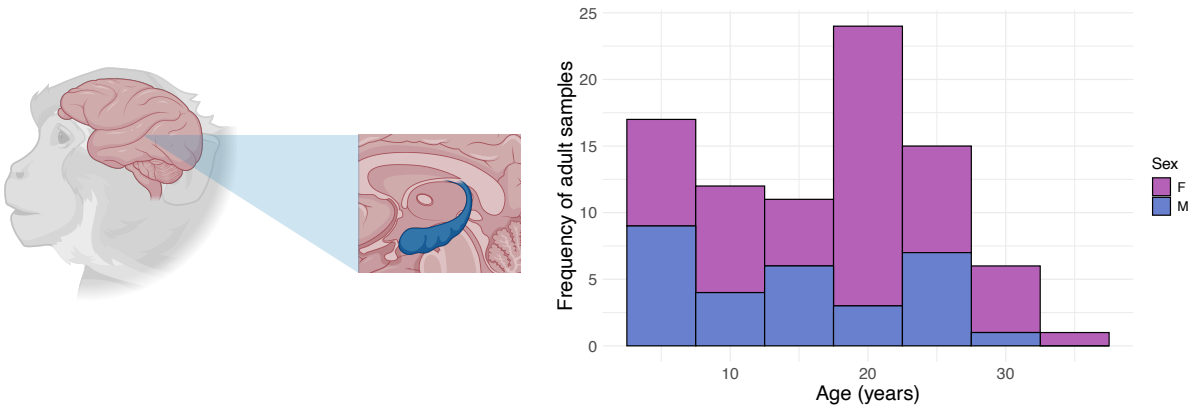


Fig. 3.1 Age distribution of the 86 rhesus macaque hippocampal samples included in the DNAm dataset. Bars represent the number of individuals within 5-year age bins and are colored by sex. The youngest individual was 3.72 years of age, indicating that all samples were collected after sexual maturity while the oldest individual was 35.03 years of age. The dataset spans the adult lifespan including representation across midlife as well as advanced ages (>20 years)

Samples were obtained from the tissue archive of the NIH NIA-funded Aging Primate Resource, housed at the Oregon National Primate Research Center (ONPRC), which followed the recommendations of the 2013 Edition of the American Veterinary Medical Association

Guidelines for the Euthanasia of Animals; tissues were collected opportunistically from veterinary culls.

3.2.2 DNA library preparation and sequencing

Combined DNA/RNA extraction from hippocampal tissue has been described previously (T. J. Anderson et al. 2025). Briefly, a combined DNA/RNA extraction protocol (Qiagen DNA/RNA Allprep kit) was used to isolate genomic DNA and total RNA by the ONPRC Primate Genetics Core. Extractions were done in groups of 24 over the course of 1 day with a randomized sample order to ensure ages and sexes were not all grouped together. Importantly, the combined extraction protocol ensured that all downstream molecular datasets were derived from the same individuals, enabling direct integration of matched DNAm and RNAseq data.

RRBS libraries were generated at the KCVI Epigenetics Consortium using established protocols previously described (Carbone et al. 2019). Briefly, genomic DNA was digested with MspI, followed by adaptor ligation, size selection, bisulfite conversion, and PCR amplification. Libraries were subjected to quality control and normalized using qPCR prior to sequencing at the Genomics & Cell Characterization Core Facility (GC3F). Sequencing was performed on an Illumina HiSeq 4000 platform to generate single-end 100 bp reads.

3.2.3 Data preprocessing

Raw RRBS reads were first assessed for quality using FastQC (Andrews 2010), followed by adapter and low-quality base trimming with TrimGalore (v0.6.10) (Martin 2011). Trim Galore was run in RRBS mode using the --rrbs flag, which enables automatic trimming of Illumina TruSeq adapters, removal of MspI digestion artifacts, and quality trimming of bases

below a Phred score of 20. Reads shorter than 20 bp after trimming were discarded by default. A second round of FastQC was performed to ensure the effectiveness of trimming. The trimmed reads were then aligned to the rhesus macaque reference genome Mmul_10 (Accession: GCA_003339765.3) using Bismark (Krueger and Andrews 2011). Alignment was performed in single-end mode with Bismark's default parameters after building the bisulfite-converted genome index. All autosomes (chromosomes 1-20) and the X chromosome were included in downstream analyses; CpGs mapping to the Y chromosome were excluded due to inconsistent coverage across sexes. All samples had mapping efficiencies above 60% (mean = 0.74 ± 0.04), consistent with high-quality alignments. Because RRBS libraries were generated using MspI digestion, which enriches for CpG-dense fragments, downstream analyses focused on CpG methylation and were not designed to comprehensively assess non-CpG methylation. Following alignment, CpG methylation profiles were extracted using Bismark's methylation extractor in single-end mode for downstream analysis.

CpG sites with ≥10× coverage in at least 80% of samples were retained using the comethyl package (Mordaunt et al. 2022). This resulted in a matrix of 802,283 CpGs that were then used in all downstream analyses. To account for unmeasured technical variation, we performed surrogate variable analysis (SVA) using the sva R package (Leek et al. 2012) on the top 10,000 most variable CpG sites (in M-value space), using a model adjusted for age and sex. From the remaining SVs, we retained SVs explaining >2% of total variance in the methylation matrix that were not significantly associated with metadata variables of interest (e.g., age and sex; FDR < 0.05) (**Fig. S3.1**). These SVs were included as covariates in the downstream methylation analyses to control for potential latent technical effects.

3.2.4 CpG-level differential methylation and variance analysis

Age-associated differentially methylated positions (age-DMPs) were identified using the bsseq (Hansen et al. 2012) and DSS (H. Feng et al. 2014) packages, applying models that accounted for relevant biological (i.e., sex) and technical covariates. Significant differential methylation at individual CpG sites was tested using DSS's multifactor model functionality (FDR < 0.1). The model examined the effect of age while including sex and technical effects as covariates to identify age-DMPs. Significant sites were classified as exhibiting increased or decreased methylation based on the direction of the test statistic (stat > 0 = methylation gain; stat < 0 = methylation loss).

CpG methylation variance analysis was conducted using the missMethyl package (Phipson et al. 2016) to identify age-associated variably methylated positions (age-VMPs). Raw methylation proportions were extracted using bsseq (Hansen et al. 2012), with boundary correction applied prior to logit transformation and conversion to M-values. Variance was assessed using the varFit function in missMethyl, which models variability by first estimating deviations from fitted mean methylation levels and then testing whether these deviations are associated with age using a linear modeling framework implemented in limma, while accounting for sex and technical covariates. Mean-variance trend adjustment was also applied. The resulting logVarRatio represents the log2 fold-change in variability associated with the age coefficient. Multiple testing correction was performed using false discovery rate (FDR), and sites with age-associated variance at FDR < 0.1 were considered significant.

3.2.5 Region-level differential methylation analysis

To assess region-level monotonic and nonlinear methylation trajectories across age, we applied autoregressive integrated moving average (ARIMA) modeling to region-level methylation data using a similar approach outlined in (T. J. Anderson et al. 2025; Márquez et al. 2020; Watowich et al. 2022) to identify age-associated DNAm regions exhibiting significant nonzero methylation trajectories. Regions were defined using the regionFinder3 function in bsseq (Hansen et al. 2012), merging CpGs, from the initial matrix of 802,283 CpGs, located within 1 kb. For each region, percent methylation was calculated as the ratio of methylated to total reads. Regions were retained if they showed moderate methylation (average between 10-90%) and complete coverage across samples resulting in a matrix of 50,578 regions that was used as input for the ARIMA modeling. Regions varied in length from 3-16,403 bp and contained a minimum of 3 CpGs with the largest spanning 1,621 CpGs. Following preprocessing, methylation data were adjusted for sex and technical effects using limma (Ritchie et al. 2015). ARIMA models were then fit to time-series data for each region, and regions exhibiting significant nonzero trends were retained using Ljung–Box residual tests. Fitted values from ARIMA models were used for clustering. To determine the optimal number of clusters, we reduced dimensionality of predicted trajectories using principal component analysis (PCA), retaining the top 50 components, and applied k-means clustering on the PCA-transformed data. The optimal number of clusters (k = 4) was determined via the gap statistic with 50 bootstrap iterations, selecting k using the "firstSEmax" criterion. Hierarchical clustering (hclust) was used to group the four distinct clusters, which were analyzed for shared temporal methylation patterns. To estimate the age at which methylation trajectories shifted in nonlinear clusters, we fit LOESS

curves to average % methylation values and identified the inflection point as the age with the lowest or highest predicted expression along the smoothed curve.

3.2.6 Annotation analysis

To facilitate biological interpretation, age-DMPs, age-VMPs and age-associated regions were annotated using ChIPseeker (G. Yu et al. 2015; Q. Wang et al. 2022). Each element was mapped to the nearest gene and assigned to functional genomic features such as promoters (±3 kb from TSS), exons, introns, or intergenic regions using the Mmul_10 reference genome (Accession: GCA_003339765.3). When a site or region overlapped multiple genomic features, ChIPseeker applied a hierarchical assignment prioritizing promoter regions first, followed by exons, introns, and intergenic regions.

3.2.7 Ontology enrichment analysis

Ontology enrichment analysis for all significant age-DMPs, age-VMPs and age-associated regions was performed using g:Profiler (Raudvere et al. 2019). Enrichment analysis was conducted for Gene Ontology (GO) Biological Processes and KEGG pathways using the default statistical domain restricted to annotated genes. Significance was determined by Benjamini-Hochberg false discovery rate correction (FDR < 0.05). To enhance specificity, the maximum term size was set to 2,500 with annotation performed using the Mmul_10 reference genome.

3.2.8 Transcription factor binding site enrichment analysis

Motif enrichment analysis was performed using HOMER v4.11 (Heinz et al. 2010) with the Mmul_10 reference genome to identify transcription factor (TF) binding motifs enriched within significant age-DMPs, age-VMPs and age-associated regions. BED files for each enrichment were analyzed with a fixed 75 bp window centered on each site/region and compared against a background set of all sites/regions tested. For all enrichments, HOMER's findMotifsGenome.pl command generated both de novo and known motif enrichments. For the purpose of this analysis, only known motif enrichments were reported in the results and biological interpretation. To ensure relevance to the *Macaca mulatta* regulatory landscape, motifs corresponding to non-mammalian transcription factors (e.g., AP2/EREBP, NAC, WRKY families) were excluded prior to downstream analysis. Significant motifs were interpreted as sequence features overrepresented near age-DMPs, age-VMPs or age-associated regions relative to background (FDR < 0.01).

3.2.9 Genic and intergenic intersection analyses

To evaluate whether age-associated DNAm regions may contribute to *cis*-regulatory control of gene expression during hippocampal aging, we performed integrative analyses designed to interrogate both gene-proximal (genic) and intergenic regulatory elements. Specifically, we asked whether age-associated methylation regions overlap genomic features capable of influencing transcription either directly through genic regulation or indirectly through distal regulatory elements such as enhancers.

To evaluate potential genic *cis*-regulatory relationships, we intersected age-associated DNAm regions annotated to genic features with differentially expressed genes (DEGs) identified

from matched hippocampal RNAseq data (T. J. Anderson et al. 2025) using ARIMA modeling. Regions overlapping DEGs were classified as candidate genic regulatory loci in which age-associated methylation dynamics may be associated with transcriptional remodeling across the adult lifespan. Although this approach does not establish direct regulatory causality, it enables identification of putative *cis*-regulatory domains linking DNAm changes to gene expression during hippocampal aging.

To evaluate potential intergenic *cis*-regulatory relationships, we investigated whether age-associated DNAm regions annotated to intergenic features overlapped known enhancers. To assess overlap with enhancer elements, we lifted macaque coordinates (Mmul_10) to the human hg38 assembly using the UCSC liftOver tool (Casper et al. 2025). Lifted query regions were intersected with the Roadmap Epigenomics chromatin state maps for human hippocampus (E071) (Kundaje et al. 2015), retaining regions overlapping active enhancer states (states 6 and 7 of the 15-state ChromHMM model). To confirm enhancer status and assign predicted gene targets, these Roadmap-identified regions were further intersected with ENCODE candidate *cis*-regulatory elements (GRCh38 ELS dataset) and putative enhancer target genes were identified using integrated enhancer-gene association datasets derived from expression quantitative trait loci (eQTL) analyses filtered for human hippocampus (J. E. Moore et al. 2026). While this approach does not establish direct regulatory causality, it enables identification of distal *cis*-regulatory elements which may shape gene expression during hippocampal aging.

We also intersected intergenic age-associated DNAm regions with transposable element (TE) annotations to determine whether regions preferentially occur within repetitive genomic contexts. We did this using annotations obtained from RepeatMasker (Smit et al. 2015) via the UCSC Table Browser (Casper et al. 2025) with the Mmul_10 reference genome. To determine

whether observed overlaps exceeded random expectation, we performed permutation-based enrichment testing using the overlapPermTest function from the regioneR package (v1.40.1) (Gel et al. 2016), employing a background set of all tested regions.

3.2.10 Promoter methylation and gene expression association analysis

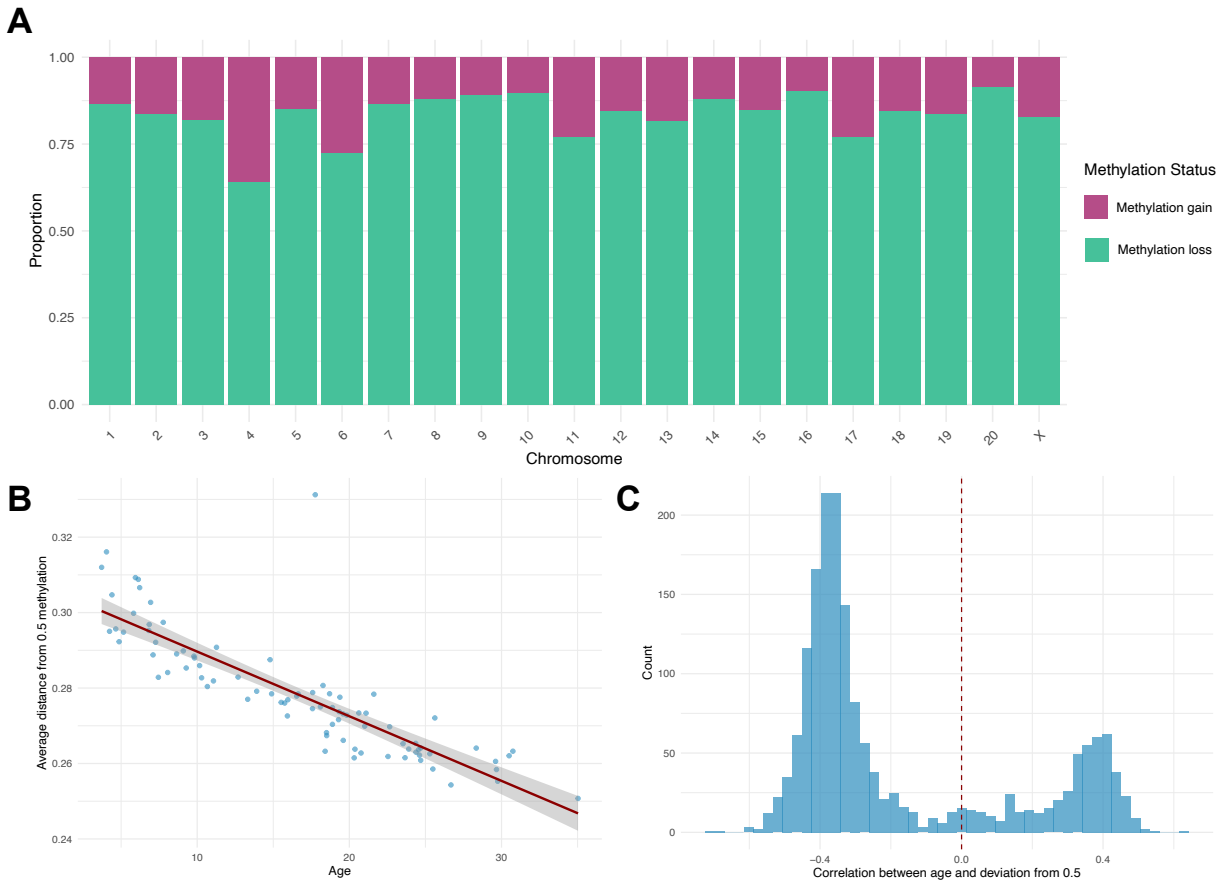
To directly evaluate whether age-associated changes in promoter DNAm predict changes in gene expression, we tested for associations between promoter methylation and residualized expression across matched individuals. Promoter regions were defined as ±2,000 bp from the transcription start site (TSS) for each DEG identified from matched hippocampal RNAseq data (T. J. Anderson et al. 2025). For each gene's promoter window, mean percent methylation across all RRBS-covered CpGs was calculated per sample using sex- and surrogate variable-adjusted methylation residuals. Of the 2,679 DEGs, 728 (27.2%) had sufficient coverage (≥1 CpG) within their TSS ±2 kb window and were retained for analysis. Expression data were residualized values generated as described previously (T. J. Anderson et al. 2025), with sex and technical covariates regressed out; these were used directly to ensure comparability with the methylation residuals. For each gene, a linear model was fit relating residualized promoter methylation to residualized expression (expression ~ promoter methylation). The sign of the regression coefficient (β) was used to classify each association as repressive ($\beta < 0$; increased methylation associated with decreased expression) or activating ($\beta > 0$). Multiple testing correction was applied using the Benjamini-Hochberg false discovery rate (FDR < 0.05). To assess whether the repressive direction was enriched beyond chance expectation, a one-sided binomial test was applied to all tested genes.

3.3 Results

3.3.1 CpG-level analyses reveal limited differences but widespread variance remodeling with age

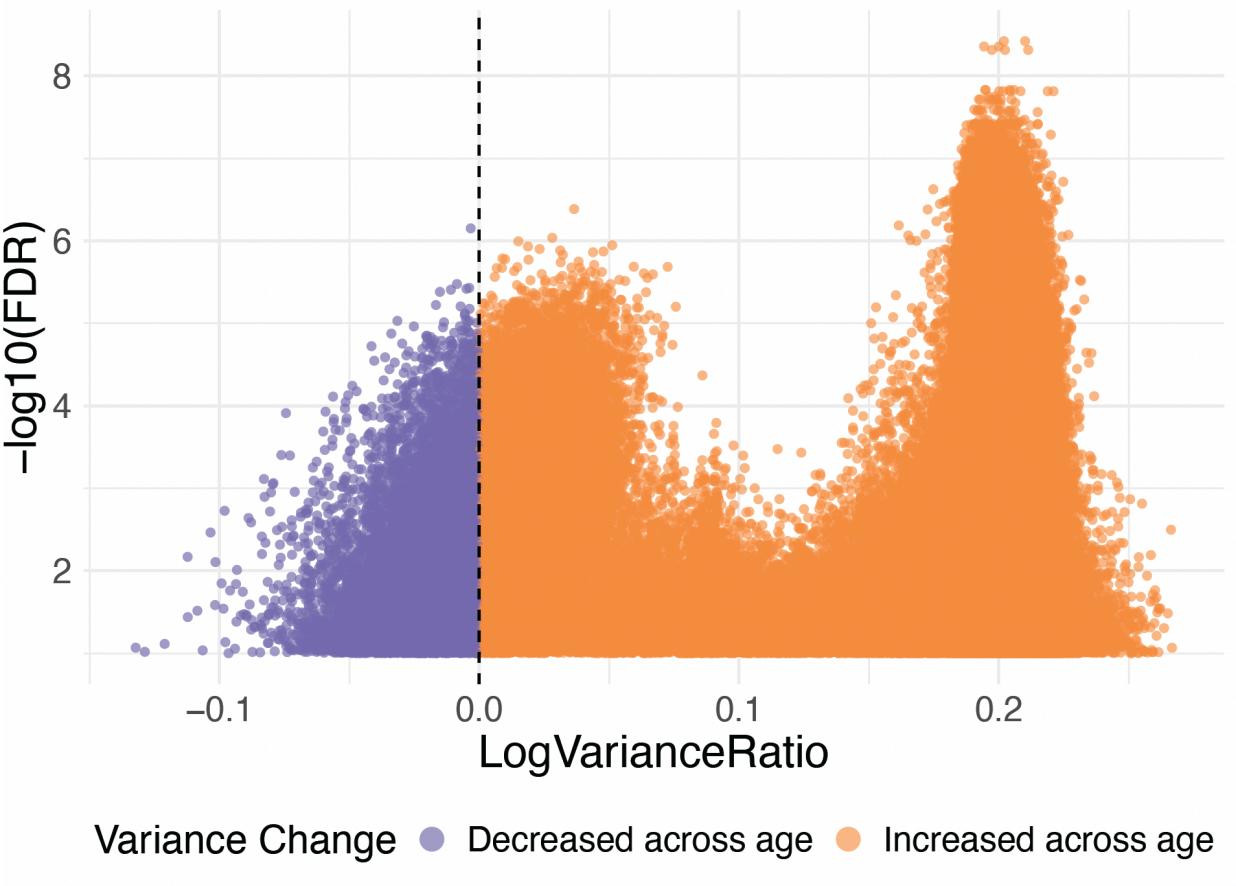
We identified 1,755 age-DMPs (~0.2% of 802,283 CpG sites tested; FDR < 0.1), indicating that relatively few CpGs exhibit consistent directional changes in mean methylation across age and of these, most exhibited a loss of methylation across the adult lifespan (84.0% age-DMPs) (**Fig. 3.2A**). Across all age-DMPs, methylation levels shifted toward intermediate values with advancing age, as quantified by a decrease in the absolute deviation from 0.5 methylation ($\beta = -0.00173$, 95% CI [−0.00194, −0.00148], $p = 2.2e{-}16$) (**Fig. 3.2B**). This pattern indicates that CpGs tend to move away from highly methylated or unmethylated states over time. Consistent with this, we observed a negative correlation between age and deviation from intermediate methylation across sites (mean $\rho = -0.18$; Wilcoxon signed-rank test, $p = 6.1e{-}81$) (**Fig. 3.2C**). This convergence toward intermediate states is consistent with previously described patterns of age-associated epigenetic drift (Seale et al. 2022; Hannum et al. 2013; Field et al. 2018). See **Supplemental Results, Discussion** and **Tables S3.2-3.6** for full description of CpG-level analyses and results.

Fig. 3.2 (next page) CpG-level differential methylation reveals global loss of methylation across age and loss of epigenetic fidelity. A) Proportional bar plot of age-DMPs (n = 1,755; FDR < 0.1; ~0.2% of total CpGs tested) across age in 86 hippocampus samples. Ordered by chromosome number and bars are colored by whether a site gains or loses methylation across age. B) Linear regression of average distance from 0.5 methylation across all age-DMPs ($\beta = -0.00173$, 95% CI [−0.00194, −0.00148], $p = 2.2e{-}16$). C) Histogram showing correlation between age and deviation from an intermediate methylation level for all age-DMPs (significant mean correlation = −0.18; Wilcoxon signed-rank test, $p = 6.1e{-}81$)



In contrast to the modest number of age-DMPs, we identified 237,531 CpG sites that exhibited differential variability in methylation (age-VMPs) (29.6% of 802,283 CpG sites tested; FDR < 0.1). As expected, these sites overwhelmingly (95.6% age-VMPs) increased in variance across age (**Fig. 3.3**). Increased variability was observed in promoters and introns of genes significantly enriched for signal transduction and neuronal processes (**Table S3.5**).

Fig. 3.3 (next page) Volcano plot of all significant age-VMPs highlights increased variance at CpG sites across the adult lifespan. Of the 237,531 total age-VMPs (FDR < 0.1; 29.6% of total CpGs tested), 227,068 age-VMPs (95.6%) increased in variance across age (mean LogVarRatio = 0.18; median = 0.20) while 10,463 (4.4%) decreased in variance across age (mean LogVarRatio = –0.03, median = –0.026)



Collectively, our CpG-level findings indicate that hippocampal DNAm aging in rhesus macaques is characterized primarily by increased stochastic variability and convergence toward intermediate methylation states rather than widespread, gains or losses at individual CpGs. These patterns are consistent with a progressive loss of epigenetic fidelity with age, similar to patterns observed in humans and other species and supporting the relevance of rhesus macaques as a model for brain aging (M. J. Jones et al. 2015; Hayflick 2007).

3.3.2 Region-level methylation trajectories highlight midlife inflections

We next assessed methylation dynamics at the region-level because CpG sites nearby one another often change together with age (Ochana et al. 2025) and this coordinated activity generally has stronger gene regulatory control than single CpG sites. We first used ARIMA models to identify genomic regions that show age-associated methylation trajectories. We identified 5,163 age-associated regions (10.2% of total regions; region length range = 5-6,088 bp, mean = 420 bp, median = 99 bp) exhibiting significant nonzero trends in methylation percent across age. These regions primarily fell into intronic (10.7% in 1st introns; 25.5% in other introns) and distal intergenic regions (30.3%) and were enriched for processes related to nervous system development (GO:0007399), regulation of cellular component organization (GO:0051128) and generation of neurons (GO:0048699); enriched KEGG pathways included endocytosis (KEGG:04144), regulation of actin cytoskeleton (KEGG:04810) and axon guidance (KEGG:04360) (**Table S3.7**). Hierarchical and k-means clustering revealed that these regions fit four broad methylation trajectories (**Fig. S3.2**). The first two clusters highlight primarily monotonic (i.e., linear) methylation trajectories while the last two highlight nonlinear methylation trajectories.

Cluster 1 contained 372 age-associated regions (7.2% of significant age-associated regions) which typically increased in methylation % across age (**Fig. 3.4A**). These regions were primarily annotated to distal intergenic (36.8%) and intronic regions (10.8% in 1st introns; 22.9% in other introns) (**Table S3.7**). There were no significantly enriched GO or KEGG ontology results and motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs.

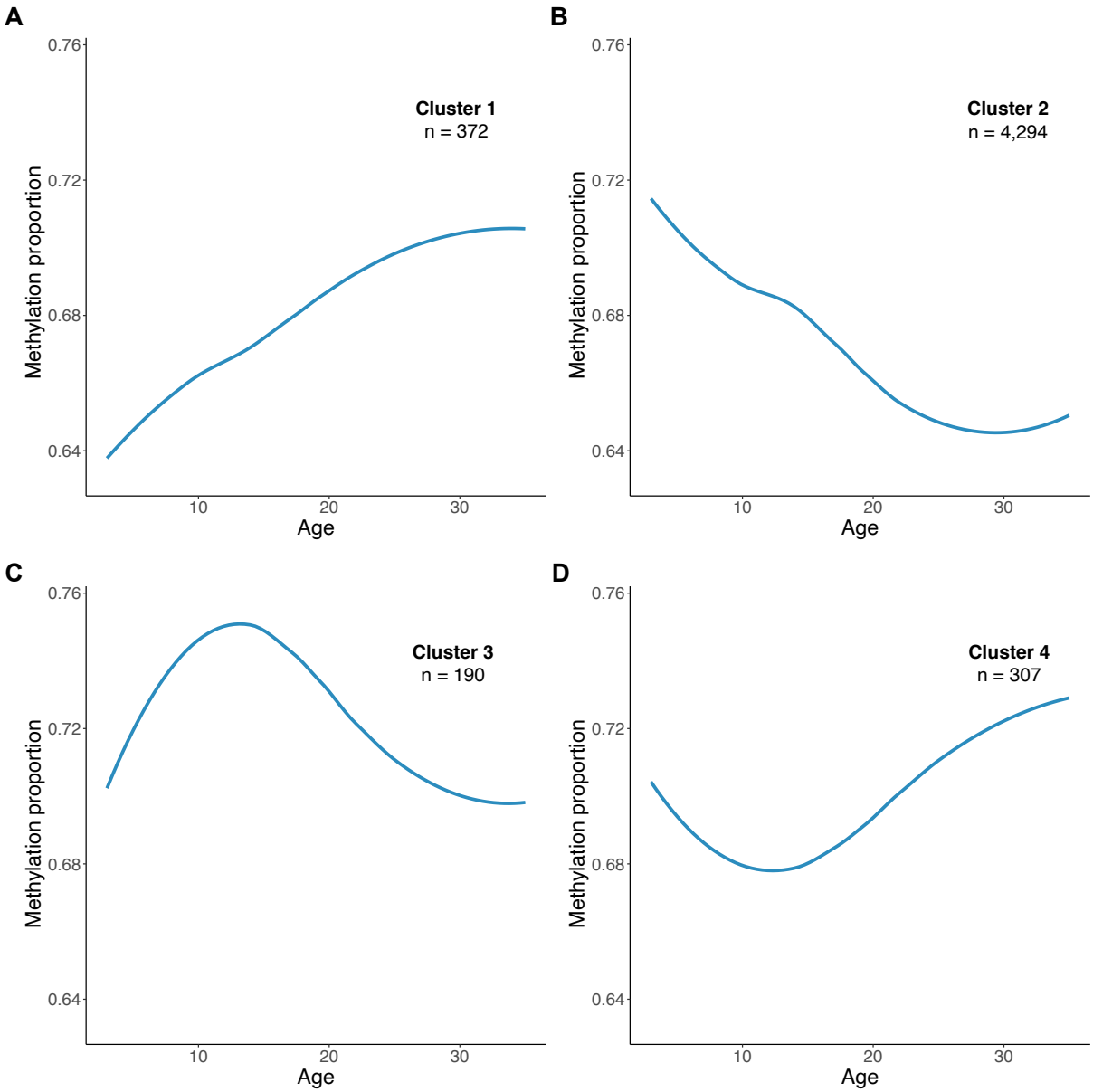


Fig. 3.4 Methylation trajectory plots for each region-based cluster exhibiting nonzero methylation trajectories across age. A-D) Mean DNA methylation trajectory (proportion) across age for each cluster of regions, identified through hierarchical clustering of ARIMA-fitted methylation values. The solid blue line represents the LOESS-smoothed average methylation trend over the adult lifespan for that cluster. Number of regions in each cluster is denoted by n

Cluster 2 contained 4,294 age-associated regions (83.2% of significant age-associated regions) which typically decreased in methylation % across age (**Fig. 3.4B**). These regions were primarily annotated to intronic (11.0% in 1st introns; 26.0% in other introns) distal intergenic regions (28.3%). GO ontology analysis identified nervous system development (GO:0007399) as

the top enriched GO term along with generation of neurons (GO:0048699) and neuron differentiation (GO:0030182) among the top enriched terms. The top 3 enriched KEGG pathways for these regions included endocytosis (KEGG:04144), phospholipase D signaling pathway (KEGG:04072) and axon guidance (KEGG:04360). Motif enrichment analysis identified significant overrepresentation of binding motifs for 25 TFs. The top enriched motif was for NeuroG2, a TF expressed in neural progenitor cells and is implicated in the differentiation and survival of midbrain dopaminergic neurons (Deng et al. 2010). Other top enriched motifs included binding sites for zinc-finger proteins (Zac1; ZBTB18; Maz; HIC1), TFs involved in neurogenesis and neuron differentiation (Ascl1; Atoh1) and oligodendrocyte formation (Olig2) (**Table S3.7**).

Cluster 3 contained 190 age-associated regions (3.7% of significant age-associated regions) that exhibited a nonlinear methylation trajectory characterized by an increase in methylation across early adulthood, reaching a maximum at 13.2 years (as estimated from the LOESS-smoothed methylation % curve) followed by a subsequent decline (**Fig. 3.4C**). These regions were primarily annotated to distal intergenic regions (36.8%) and intronic regions (8.4% in 1st introns; 26.8% in other introns). The top 3 enriched GO terms for regions in this cluster included vesicle transport along microtubule (GO:0047496), vesicle localization (GO:0051648) and vesicle cytoskeletal trafficking (GO:0099518) (**Table S3.7**). These processes are central to intracellular trafficking, a known hallmark of aging (López-Otín et al. 2023), and synaptic communication, suggesting that midlife remodeling of methylation at these loci may reflect age-associated changes in cellular transport dynamics. There were no significantly enriched KEGG pathways in this cluster and motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs.

Cluster 4 contained 307 age-associated regions (5.9% of significant age-associated regions) that exhibited a nonlinear methylation trajectory characterized by a decrease in methylation across early adulthood, reaching a minimum at 12.3 years (as estimated from the LOESS-smoothed methylation % curve) followed by a progressive increase in methylation (**Fig. 3.4D**). These regions were primarily annotated to distal intergenic (45.6%) and intronic regions (8.1% in 1st introns; 21.8% in other introns). There were 2 significantly enriched GO terms for the regions in this cluster (intracellular signal transduction [GO:0035556]; embryonic morphogenesis [GO:0048598]) and 1 KEGG pathway (type II diabetes mellitus [KEGG:04930]) (**Table S3.7**). These processes are central to intracellular signaling, again a recognized hallmark of aging (López-Otín et al. 2023), and developmental patterning, suggesting that nonlinear remodeling of methylation at these loci may reflect age-associated changes in intercellular communication. Motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs.

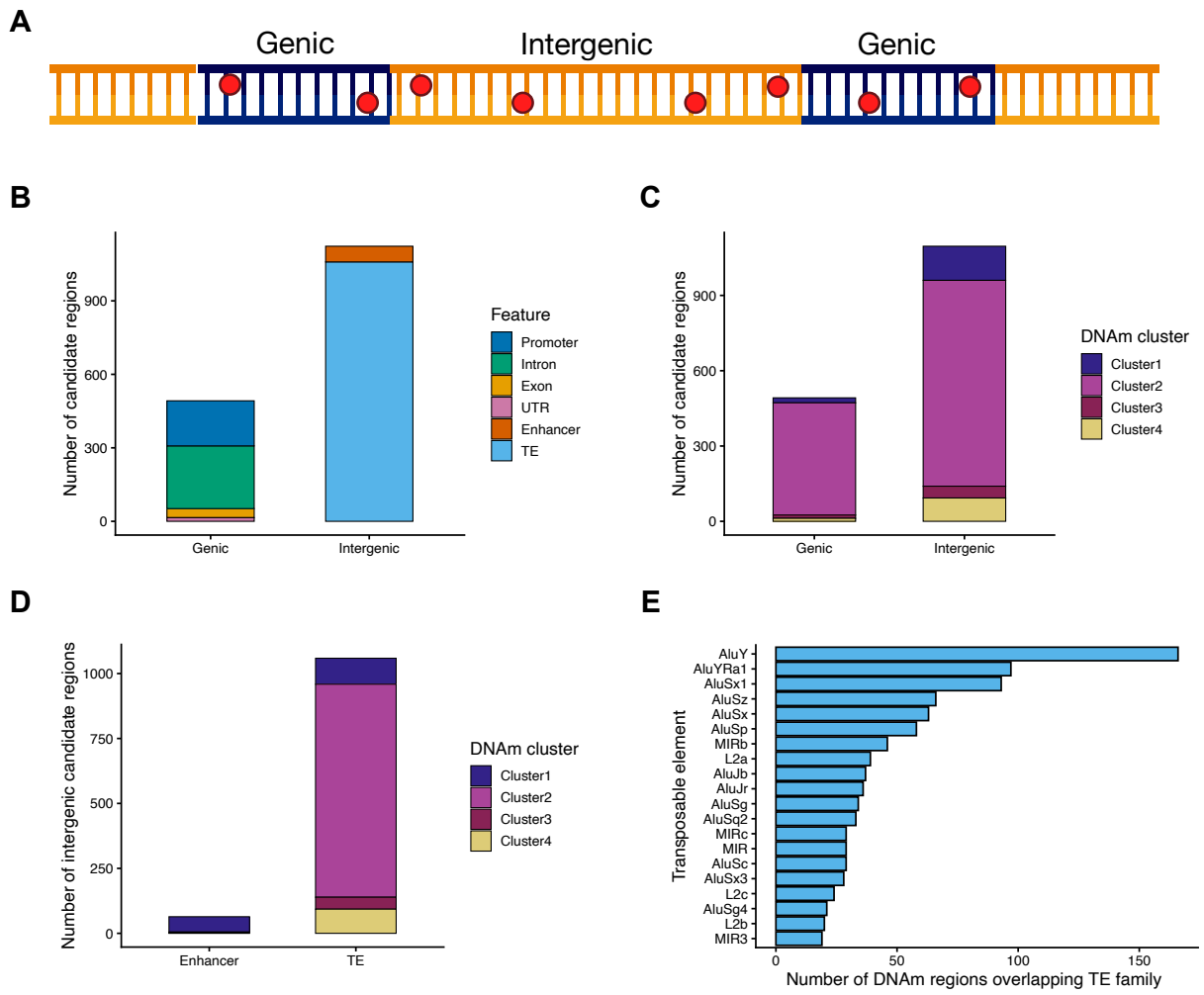
Given prior evidence of widespread sex-specific patterns in DNA methylation aging (Masser et al. 2017; Chinn et al. 2021), we next examined whether the molecular signatures of age-associated methylation change differed between females (n = 56) and males (n = 30) by repeating our analyses within sex. When looking within sex, we identified 3,836 age-associated regions in females (60.5% overlap with original ARIMA approach) and 2,758 regions in males (41.4% overlap with original ARIMA approach) (**Table S3.8**). Similar to the original ARIMA approach, hierarchical and k-means clustering revealed that age-associated regions for both females and males fit 4 broad methylation trajectories. Despite moderate overlap with the original approach for both sexes, there were very few conserved regions when comparing across sex (n = 416; 10.8% overlap). Importantly, this pattern was not attributable solely to X

chromosome effects. Although females and males exhibited a comparable number of X-linked age-associated regions (122 and 115, respectively), only 8 were shared, demonstrating that sex-specific methylation trajectories occur broadly across the genome, including both autosomes and the X chromosome. Functional annotation of sex-specific age-associated regions revealed broadly similar genomic distributions and enrichment patterns in both females and males, recapitulating the overall analysis (See **Supplemental Results** and **Table S3.8**).

3.3.3 Genic DNAm remodeling intersects age-associated transcriptional change

DNAm modifications can occur both within genic and intergenic genomic contexts (**Fig. 3.5A**). To determine whether age-associated DNAm changes within genic features were associated with age-dependent transcriptional remodeling, we intersected age-associated DNAm regions annotated to genic features (e.g., promoters, introns, exons) with DEGs identified from matched hippocampal RNAseq data using ARIMA modeling (T. J. Anderson et al. 2025). Regions that overlapped or were annotated to DEGs were classified as candidate genic *cis*-regulatory loci, representing sites where age-associated methylation dynamics may be linked to changes in gene expression across the adult lifespan.

Fig. 3.5 (next page) Genic and intergenic intersection analyses highlight overlap with *cis*-regulatory elements. A) Conceptual overview of how DNAm can operate in both genic and intergenic genomic contexts. Red circles represent methylated CpG sites (DNAm) occurring along the DNA sequence. Genic regions include promoters, introns, exons, and untranslated regions within gene bodies, whereas intergenic regions may intersect distal regulatory elements such as enhancers. B) Distribution of candidate genic and intergenic regulatory regions identified through intersection analyses. Bars show the number of age-associated DNAm regions overlapping genic features (promoters, introns, exons, and UTRs) or intergenic regulatory elements (enhancers and TEs). C) Distribution of candidate regions by DNA methylation trajectory cluster. Bars indicate the number of genic and intergenic candidate regions derived from each DNAm trajectory cluster identified through ARIMA modeling. D) Distribution of intergenic candidate regulatory regions by DNAm cluster for regions overlapping annotated enhancers and TEs. E) Most frequently intersecting TEs among intergenic DNAm regions. Bars show the number of DNAm regions overlapping TEs



The intersection analysis yielded 492 genic DNAm regions annotated to DEGs (**Table S3.9**). Of these, multiple regions (n = 125) were annotated to the same gene, resulting in 367 unique gene targets. DNAm region lengths ranged from 10 bp to 6,088 bp (mean = 408 bp; median = 99 bp). The majority of these regions overlapped DEG introns (15.9% in first introns; 28.4% in other introns) and promoters (≤3 kb; 31.9%) features of DEGs (**Fig. 3.5B**). Most candidate genic regions were derived from Cluster 2 (90.9%), the largest methylation cluster characterized by decreases in methylation percent across the adult lifespan (**Fig. 3.4B** and **3.5C**), whereas only a small proportion originated from Clusters 3 and 4 (5.1%), which exhibited

nonlinear midlife trajectories (**Fig. 3.4C&D** and **3.5C**). The corresponding DEG targets, as previously reported (T. J. Anderson et al. 2025), displayed both monotonic and nonlinear expression trajectories, including age-associated up- or downregulation as well as inflection points centered around ~10 years.

Collectively, these genic DNAm regions represent candidate *cis*-regulatory loci in which age-associated methylation remodeling coincides with transcriptional change in the macaque hippocampus. Although these intersections do not establish direct regulatory causality, they identify specific genomic regions for functional validation. Notably, several candidate regions overlap genes previously implicated in brain aging and neurodegenerative disease, underscoring their potential biological relevance.

3.3.4 Intergenic age-associated regions overlap known enhancers

To assess whether age-associated DNAm changes in intergenic regions may have *cis*-regulatory relevance, we evaluated overlap between intergenic DNAm regions and annotated enhancer elements. Because comprehensive macaque-specific brain enhancer annotations remain limited for recent macaque genome assemblies, we lifted macaque intergenic DNAm regions to the human hg38 assembly to evaluate overlap with annotated human enhancer elements. Of the 1,563 distal intergenic age-associated regions, 594 uniquely mapped to human coordinates and were retained for downstream analysis (multimapping regions were excluded). Among these, 64 unique regions overlapped Roadmap Epigenomics active enhancer chromatin states for human hippocampus (Kundaje et al. 2015), representing candidate enhancer-associated loci (**Fig. 3.5B** and **Table S3.10**). Of these, 44 were further confirmed to overlap ENCODE candidate *cis*-

regulatory elements with documented enhancer activity in human hippocampus (J. E. Moore et al. 2026).

Enhancer-overlapping regions were heavily concentrated in Cluster 1 (n = 59/64, 92.2%), the cluster characterized by progressive age-associated methylation gains, with only 3 enhancers derived from Cluster 2 regions (age-associated loss of methylation) and 1 each in Clusters 3 and 4 (nonlinear age-associated methylation). None of these elements had documented gene associations in the ENCODE Brain Hippocampus eQTL database (J. E. Moore et al. 2026), limiting our ability to infer the functional consequences of age-associated methylation at these enhancer regions, and the requirement for unique LiftOver precluded formal enrichment testing. Nevertheless, these findings indicate that age-associated DNAm remodeling extends beyond gene bodies to encompass distal regulatory elements, with potential implications for transcriptional remodeling during hippocampal aging.

3.3.5 Intergenic age-associated regions overlap known transposable elements

We next assessed whether intergenic age-associated regions overlapped annotated transposable elements (TEs) to determine whether these regions preferentially occur within repetitive genomic contexts. Of the 1,563 age-associated regions annotated to distal intergenic features, 1,059 overlapped annotated macaque transposable elements (TEs) (**Fig. 3.5B** and **Table S3.10**). Permutation-based enrichment testing indicated that TE overlap occurred more frequently than expected by chance ($Z = 2.41$, $p = 0.02$, 100 permutations). Across these overlaps, 1,059 unique intergenic regions intersected 418 distinct TE families. While most regions overlapped a single TE, 397 regions overlapped more than one TE (mean = 1.8 TEs per region; maximum = 12), indicating that some age-associated loci reside within TE-dense

genomic intervals. The majority of TE-overlapping regions were derived from Cluster 2 (77.4%), the largest methylation cluster characterized by decreases in methylation % across the adult lifespan (**Fig. 3.4B** and **3.5D**), with smaller contributions from Clusters 1 (9.4%), 3 (4.4%) and 4 (8.8%), indicating that TE-associated methylation changes are not restricted to monotonic trajectories but occur across multiple aging patterns (**Fig. 3.5D**). Most intersecting elements belonged to SINE and LINE families, consistent with the composition of primate genomes and Alu elements were the most abundant overlapping TE class (**Fig. 3.5E**).

3.3.6 Promoter methylation is associated with expression in a subset of DEGs

The preceding genic intersection analysis identified candidate *cis*-regulatory loci based on the co-occurrence of age-associated methylation regions and DEGs but did not directly test whether methylation levels at these loci are associated with variation in gene expression across individuals. To more directly evaluate the functional relationship between promoter DNAm and transcriptional output, we examined correlations between mean promoter methylation and gene expression across all matched samples for 728 DEGs with RRBS coverage in their promoter window. Across all tested genes, 52.9% showed a negative (repressive) relationship between promoter methylation and expression, consistent with the canonical repressive role of promoter methylation, although the directional enrichment was not significant (binomial test, $p = 0.064$). Two genes reached statistical significance after FDR correction (FDR < 0.05), both exhibiting repressive relationships: *SERPINA1* ($\beta = -4.16$, $R^2 = 0.19$, FDR = 0.013) and *GJB1* ($\beta = -4.41$, $R^2 = 0.18$, FDR = 0.013) (**Fig. 3.6**). For both genes, higher promoter methylation was associated with lower expression across individuals, accounting for ~18-19% of the variance in expression.

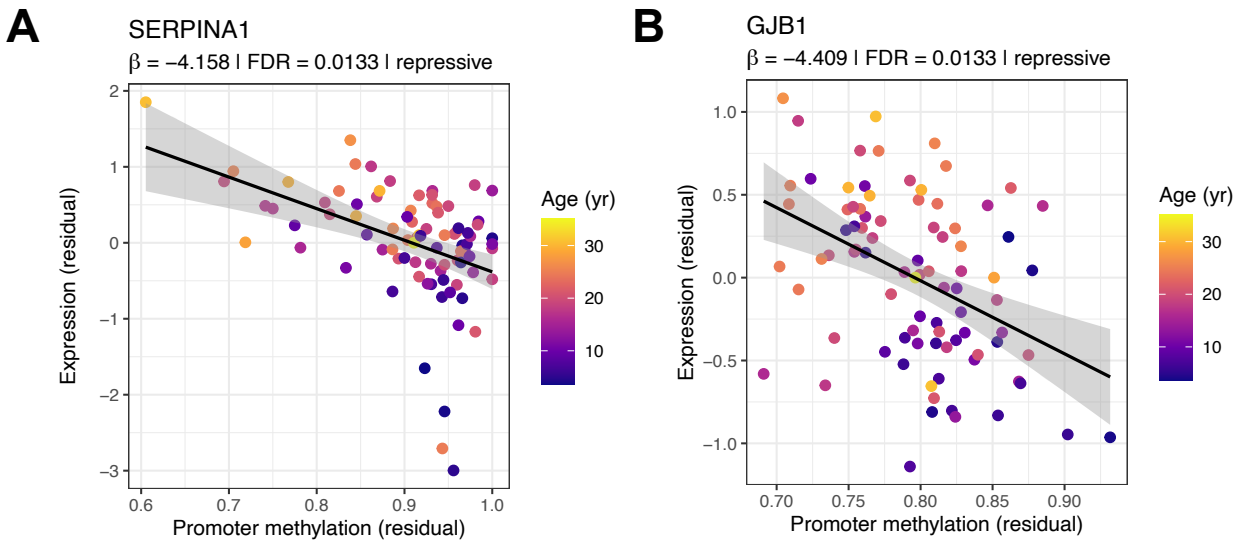


Fig. 3.6 Genes exhibiting significant repressive associations between promoter methylation and gene expression. A-B) Scatter plots of residualized promoter methylation against residualized gene expression for *SERPINA1* and *GJB1*, the two genes with significant inverse relationships between promoter methylation and expression after FDR correction (FDR < 0.05). Each point represents one individual, colored by chronological age. Promoter methylation was defined as mean percent methylation across CpGs within a ±2 kb window of the transcription start site. Beta coefficients and FDR-corrected p-values are shown for each gene

3.4 Discussion

Our study provides a comprehensive characterization of DNAm dynamics across the adult lifespan in the rhesus macaque hippocampus and reveals that aging is marked by both increased epigenetic variability at individual CpGs and shifts in methylation across genomic regions. Together, these patterns point to a progressive decline in epigenetic fidelity with advancing age, coupled with structured reorganization of regulatory loci. Region-level modeling identified both monotonic and nonlinear age-associated methylation trajectories, including a reproducible midlife inflection centered around ~10 years of age, suggesting that hippocampal aging involves not only progressive directional change but also distinct molecular transition periods that may shape later-life trajectories. Importantly, these age-associated differentially methylated regions span both genic and intergenic regulatory features, including promoters, introns and enhancer domains, indicating that epigenetic aging disproportionately affects *cis*-

regulatory elements and may contribute to age-associated transcriptional change. Comprehensive molecular characterization of brain aging across the lifespan in long-lived nonhuman primates remains limited, particularly at the level of gene regulation. By leveraging a hippocampal dataset that spans adulthood in rhesus macaques, a well-established translational model of human aging (Chiou et al. 2020), this study helps bridge the gap between rodent models and human studies and provides a framework for understanding how regulatory precision shifts over time in a brain region central to learning and memory.

Our age-DMPs, and age-associated regions, highlight shifts in which methylation levels are increased or decreased across age while age-VMPs highlight inter-individual variability associated with increased age. Collectively this highlights the following, aging does not cause dramatic changes in mean methylation at the CpG level – as evidenced by our age-DMPs. Rather, when looking at an individual CpG, our age-VMPs show that increased age is more commonly associated with heightened variability in methylation across individuals, rather than uniform gains or losses at specific CpGs. At the region level, shifts in methylation trajectories across age are more prevalent than at the single-CpG level. This is expected, as neighboring CpGs often display correlated methylation patterns, making regional analyses more sensitive to biologically coherent age-associated changes (Ochana et al. 2025). Furthermore, region-level changes were concentrated within genic features of DEGs (T. J. Anderson et al. 2025) and intergenic regions that frequently overlap enhancer elements, reinforcing the idea that remodeling of age-associated methylation disproportionately affects regulatory features of the genome. This pattern supports a model in which aging reshapes loci with regulatory potential and motivates future work aimed at disentangling how these methylation shifts influence transcriptional dynamics and functional outcomes across the adult lifespan in the primate brain.

Our findings also underscore the value of integrating both CpG- and region-level analyses when characterizing the aging methylome, a need explicitly highlighted by (Seale et al. 2022), who called for multi-scale approaches to fully understand how aging impacts epigenetic regulation.

We previously reported a midlife transition in hippocampal gene expression trajectories in rhesus macaques (T. J. Anderson et al. 2025), and here we observe a comparable shift in DNAm. Regions in Clusters 3 and 4 exhibited nonlinear trajectories characterized by early-life increases or decreases in methylation followed by directional reversals in midlife, with inflection points occurring shortly after ~10 years of age (**Fig. 3.4C** and **D**). Although evidence of this transition was most pronounced in the nonlinear clusters, subtle deviations from monotonic trends were also detectable across other clusters (**Fig. 3.4A** and **B**), suggesting that hippocampal aging may involve a broader reorganization of regulatory programs during this midlife window rather than a strictly linear progression. While functional enrichment across nonlinear clusters was modest, we observed enrichment for processes related to intracellular trafficking, vesicle transport, and intercellular communication (**Table S3.7**), which are central to synaptic function and recognized hallmarks of aging (López-Otín et al. 2023). The only significantly enriched KEGG pathway among nonlinear regions was type II diabetes mellitus (KEGG:04930), raising the possibility that insulin-resistance-related mechanisms may intersect with nonlinear DNAm trajectories and hippocampal function. Indeed, molecular alterations in insulin resistance pathways within the hippocampus have been shown to impact synaptic plasticity (Spinelli et al. 2019). However, given the limited breadth of enrichment and absence of strong motif signals, the biological underpinnings of these inflection points remain unclear, and regions exhibiting nonlinear trajectories may represent functionally heterogeneous or incompletely annotated regulatory loci.

To evaluate the regulatory potential of age-associated DNAm regions, we intersected genic regions with DEGs identified from matched hippocampal RNAseq data (T. J. Anderson et al. 2025), identifying candidate *cis*-regulatory loci where age-associated methylation may be linked to transcriptional changes (**Table S3.9**). These candidate regions highlight genes with established roles in neural maintenance and disease susceptibility. For example, age-associated region chr7:77879817–77879847 overlaps the first intron of *IGF1R* and exhibits an overall decline in methylation across the adult lifespan, though this trajectory is not strictly monotonic – methylation first increases through early-to-mid adulthood before declining more markedly in later life (after ~10 years) (**Fig. S3.3**). *IGF1R* expression, in turn, shows a broadly inverse pattern: expression increases across early adulthood and is sustained through midlife, with further increases in expression in the oldest individuals (**Fig. S3.4**). While the relationship between first intron methylation and gene expression has historically received less attention than promoter methylation, an inverse relationship between first intron methylation and gene expression has been documented genome-wide across vertebrate tissues and species (Anastasiadi et al. 2018), and this broadly inverse pattern at the *IGF1R* first intron is consistent with a potential role for genic methylation in modulating transcription across the adult lifespan. The biological significance of this pattern may depend on the timing of these changes. *IGF1R* expression persists in neurogenic niches such as the dentate gyrus, consistent with its role in promoting neurogenesis (Wrigley et al. 2017; M. F. Anderson et al. 2002), and because hippocampal neurogenesis extends into adulthood (Eriksson et al. 1998; Roy et al. 2000; Disouky et al. 2026), sustained expression through midlife may reflect this ongoing activity in this region. However, the relationship between *IGF1R* expression and aging is complex: elevated *IGF1R* expression has been associated with reduced longevity in rodents when looking across

heart, lung, kidney and brain tissue (Azpurua et al. 2013), and reduced IGF signaling is linked to extended lifespan in humans carrying function-impairing *IGF1R* variants when examining Ashkenazi Jewish centenarians venous blood samples (Suh et al. 2008; Wrigley et al. 2017). IGF signaling dysregulation has also been implicated in neurodegenerative disease and brain insulin resistance (Talbot et al. 2012; Lewitt and Boyd 2024). Thus, the age-associated decline in methylation in this region – and its broadly inverse relationship with *IGF1R* expression – may intersect with pathways central to both neural maintenance and disease susceptibility.

To more directly test whether promoter methylation predicts transcriptional output, we examined associations between promoter methylation and expression across matched individuals for 728 DEGs with RRBS promoter coverage. The overall directional trend was consistent with the canonical repressive role of promoter methylation (L. D. Moore et al. 2013), though it did not reach statistical significance (binomial test $p = 0.064$), suggesting that promoter methylation is one of several regulatory inputs shaping expression. Two genes reached significance after FDR correction – *SERPINA1* and *GJB1* – both showing repressive relationships that explained ~18-19% of expression variance (**Fig. 3.6**). *SERPINA1* encodes alpha-1-antitrypsin, a serine protease inhibitor with an inflammatory role that has increasingly been demonstrated to play an important role in shaping susceptibility to neurodegeneration and disease (Gollin et al. 1992; Ebbert et al. 2017; Abu-Rumeileh et al. 2020). *GJB1* encodes Connexin 32, an oligodendrocyte-expressed gap junction protein whose age-associated dysregulation or mutations have been implicated in the development of X-linked Charcot-Marie-Tooth Disease, a peripheral neuropathy (Chu et al. 2022). More broadly, connexin-mediated gap junction signaling plays a critical role in oligodendrocyte function and myelin maintenance in the central nervous system, and age-associated disruption of glial gap junction networks has been implicated in white matter

deterioration and cognitive decline (Papaneophytou et al. 2019). Notably, neither gene was flagged in the genic ARIMA intersection analysis, which can be explained given that the two approaches address fundamentally different questions: the intersection analysis identifies loci where both methylation and expression follow coordinated age-associated trajectories with input regions needing to have been identified in ARIMA modeling, while the promoter correlation analysis asks whether the variation in promoter methylation level co-varies with expression variation without requiring the promoter to exhibit a statistically significant age trajectory. That these analyses detect differing gene sets underscores the value of multi-scale integrative approaches for fully characterizing how age-associated methylation remodeling influences transcriptional dynamics in the aging hippocampus.

The observed pattern of primarily methylation gains in age-associated intergenic regions which overlap enhancer elements may suggest that methylation could be playing a role in diminishing age-related enhancer activity. Indeed, this finding corresponds with arguments that DNAm and histone modifications likely play a role in changing chromatin accessibility from an accessible to repressive state in mammalian brain aging (S. et al. 2013). However, interpreting relationships between enhancer methylation dynamics and target gene expression can be challenging, as enhancer activity reflects the combined influence of transcription factor binding, chromatin accessibility, and three-dimensional genome organization (Barral and Déjardin 2023); consequently, DNAm dynamics at enhancer elements do not always correspond directly with transcriptional activation or repression.

Although the precise regulatory relationships between methylation and gene expression require functional validation and cannot be resolved here, the findings and candidates described here represent promising targets for future functional investigation. In addition, we describe

additional candidate regions of potential interest in the **Supplemental Discussion** and **Fig. S3.5-7**.

The enrichment of age-associated intergenic regions within transposable elements further suggests that methylation remodeling is not confined to canonical regulatory features but extends into repetitive genomic contexts, consistent with the known role of DNAm in TE silencing. The observation that the majority of TE-overlapping DNAm regions were derived from Cluster 2 (age-associated loss of methylation) could suggest that DNAm erosion plays a role in TE activation which is associated with increased age is some genomic contexts (Peze-Heidsieck et al. 2022; Merenciano et al. 2025) (See **Supplemental Discussion** for more details).

Several limitations should be considered when interpreting these findings. First, this study is based on cross-sectional hippocampal samples, limiting our ability to directly infer within-individual epigenetic trajectories across the adult lifespan. Although region-level modeling provides insight into age-associated dynamics, longitudinal sampling would be necessary to determine whether observed nonlinear shifts and late-life deviations reflect true within-individual remodeling or population-level heterogeneity, but longitudinal sampling of primate brains is not possible. Second, DNAm profiles were generated from bulk hippocampal tissue. While this approach captures global aging patterns, it precludes resolution of cell-type-specific regulatory dynamics as well as subregional variation in cell types. The observed changes therefore likely reflect a combination of cell-intrinsic epigenetic remodeling and shifts in cellular composition across age. Future studies integrating single-cell or single-nucleus methylation profiling, alongside complementary epigenomic approaches such as chromatin accessibility or histone modification mapping, will be critical for resolving the cellular context of candidate regulatory regions identified here. Third, the absence of matched behavioral or cognitive

measures limits our ability to directly link epigenetic remodeling to functional outcomes. Finally, samples were collected over an extended time span (~25 years), which may introduce additional sources of variability despite standardized processing and quality control. Nevertheless, all samples were derived from neuropathologically normal individuals, and the distribution of ages represented in this dataset provides a rare opportunity to examine molecular signatures of aging in a long-lived nonhuman primate.

This study provides a comprehensive characterization of DNAm dynamics across the adult lifespan in the rhesus macaque hippocampus, demonstrating that aging in this brain region reflects both progressive increases in epigenetic variability and remodeling across regulatory loci. Rather than dramatic shifts in mean CpG methylation alone, hippocampal aging is characterized by region-level methylation dynamics that intersect genic features of differentially expressed genes and distal intergenic elements linked to recognized hallmarks of aging. These findings suggest that aging directly impacts *cis*-regulatory elements, with potential implications for transcriptional precision and neuronal function. Consistent with this, direct testing of promoter methylation-expression relationships identified repressive associations for *SERPINA1* and *GJB1* – genes implicated in neuroinflammation and glial function respectively – providing support that age-associated methylation remodeling at specific loci may be functionally linked to transcriptional output in the aging hippocampus. The identification of a reproducible midlife transition, also observed in matched gene expression data (T. J. Anderson et al. 2025), further supports a model in which hippocampal aging proceeds through distinct molecular phases rather than a strictly linear trajectory. By leveraging an adult lifespan-spanning dataset from a nonhuman primate, this work bridges rodent and human studies and establishes a framework for investigating how regulatory fidelity shifts over time in the aging brain. Together, these results

provide a foundation for future studies aimed at disentangling the causal relationships between epigenetic remodeling, gene regulation, and cognitive aging, and offer a platform for evaluating how environmental, metabolic, or therapeutic interventions may alter the trajectory of epigenetic aging in hippocampal tissue.

Bridge to Chapter IV

Chapter 3 demonstrated that hippocampal epigenetic aging in rhesus macaques is characterized not by dramatic shifts in mean DNAm at individual CpGs, but by widespread increases in epigenetic variability and region-level remodeling of potential regulatory loci – including a midlife transition that parallels the transcriptional patterns identified in Chapter 2. Together, these findings suggest that aging reshapes the regulatory landscape of the hippocampus across multiple molecular layers in a coordinated fashion. Yet gene expression is subject to regulatory control not only at the level of chromatin and transcription, but also post-transcriptionally through mechanisms that modulate whether and how efficiently transcripts are translated into functional protein. Chapter 4 addresses this additional layer of regulation by characterizing age-associated miRNA expression in the rhesus macaque hippocampus across the adult lifespan. By integrating miRNA profiles with matched transcriptomic data from the same individuals, Chapter 4 asks whether post-transcriptional regulatory dynamics mirror the transcriptional and epigenetic aging signatures identified in previous chapters and identifies candidate miRNA-gene regulatory relationships with potential relevance to brain aging and neurodegenerative disease susceptibility.

CHAPTER IV

AGE-ASSOCIATED MICRORNA EXPRESSION IN THE RHESUS MACAQUE HIPPOCAMPUS IDENTIFIES CANDIDATE POST-TRANSCRIPTIONAL REGULATORS OF BRAIN AGING

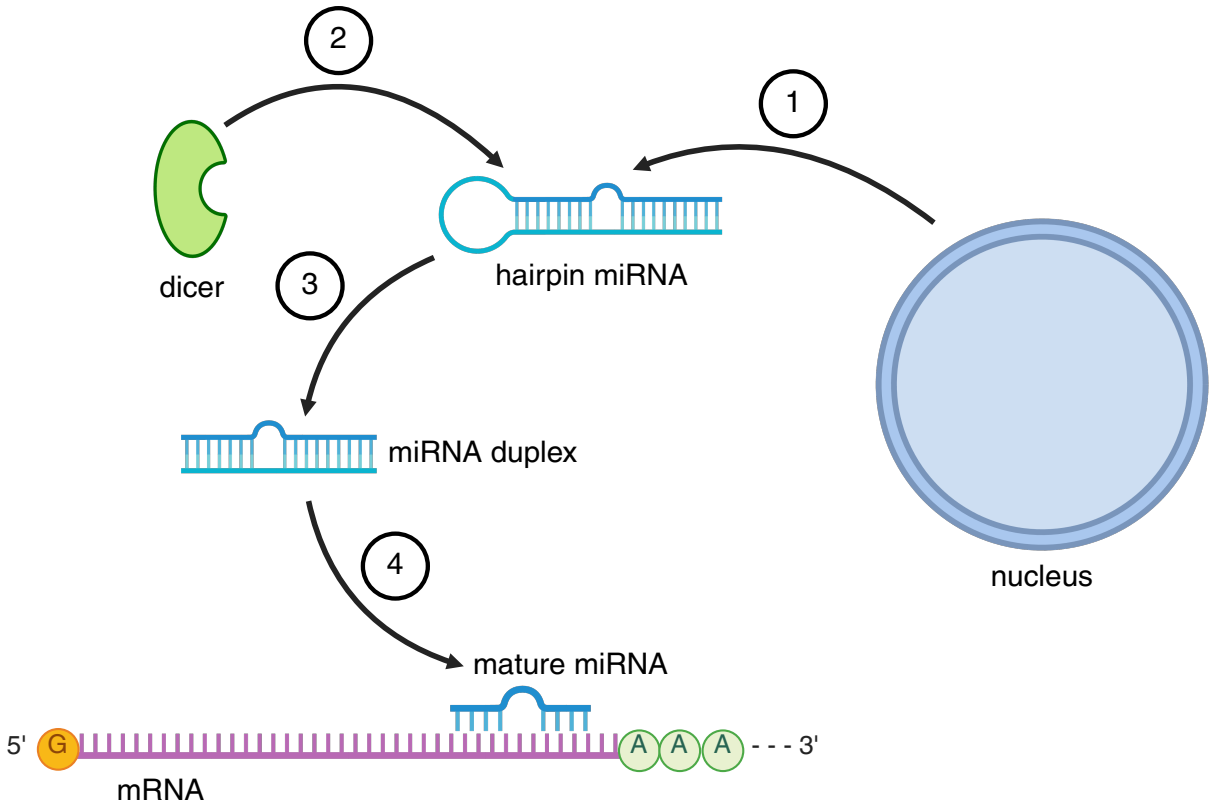
4.1 Introduction

The genomes of complex organisms encode a diverse repertoire of small noncoding RNAs (sncRNAs) that collectively exert widespread influence over gene expression, RNA processing, and translational output. Small noncoding RNAs are broadly classified into housekeeping classes, including small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), and regulatory classes, including microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) (Gou et al. 2023). Beyond these categories, advances in next-generation sequencing have revealed an expanding universe of small RNA species derived from the processing of larger precursor molecules, including tRNA-derived fragments (tRFs) and rRNA-derived small RNAs, many of which are metabolically stable and whose production is conserved across species (Wajahat et al. 2021). Despite this growing recognition, the biological roles of most small RNA classes remain incompletely characterized, and their dynamics in the context of aging, particularly in the brain, are poorly understood.

Among small noncoding RNAs, miRNAs are the most extensively studied and represent the primary focus of this work. miRNAs are short (~22 nucleotide) noncoding RNAs that regulate gene expression at the post-transcriptional level through sequence-specific interactions with target mRNAs. They are generated through a multistep biogenesis process in which primary miRNA transcripts fold into hairpin structures, are exported from the nucleus, and are

subsequently processed by the enzyme Dicer into a short RNA duplex; one strand of this duplex is incorporated into a protein complex to form the mature, functional miRNA, while the opposing strand is typically degraded. The mature miRNA guides this complex to complementary target transcripts, most often within the 3′ untranslated region, where it can promote mRNA degradation, induce transcript cleavage, or inhibit translation by preventing ribosomal engagement (**Fig. 4.1**) (Bartel 2004, 2018). Through these mechanisms, miRNAs act as modulators of protein production, refining the relationship between transcript abundance and functional protein output. A single miRNA can influence hundreds of target transcripts, often within shared biological pathways, enabling coordinated regulation of complex cellular processes (Somel et al. 2013). This regulatory capacity is particularly relevant in the hippocampus, a brain region especially sensitive to aging and whose hallmarks – including elevated oxidative stress, inflammatory pathway activation, and blood-brain barrier disruption – are thought to arise in part from coordinated changes in gene regulatory systems across the lifespan (Finch and Austad 2012; Montagne et al. 2015; Bettio et al. 2017; López-Otín et al. 2023). In the hippocampus, spatially localized translation supports synaptic plasticity and memory formation, and miRNAs are integral to activity-dependent control of protein synthesis and maintenance of neuronal homeostasis (Aakalu et al. 2001; Fernandez-Moya et al. 2014).

Fig. 4.1 (next page) Overview of miRNA production and function. 1) A miRNA is transcribed and folds back on itself to form characteristic hairpin structure; it is then transported out of the nucleus and into the cell body. 2) The enzyme dicer then trims the hairpin loop leaving a miRNA duplex molecule. 3) One strand joins part of a protein complex to form the mature miRNA molecule, while the complementary passenger strand is released and degraded. 4) The miRNA finds its complementary target mRNA strand and silences it by one of three mechanisms: causing cleavage of the mRNA strand, destabilizes mRNA by shortening polyA tail which causes early degradation, causes less efficient translation by ribosomes



Accumulating evidence indicates that miRNA expression is dynamically remodeled with age and contributes to multiple dimensions of this process, including synaptic function, inflammation, metabolic regulation, and mitochondrial activity (Che et al. 2014; Danka Mohammed et al. 2016, 2017). In rodents, numerous hippocampal miRNAs exhibit age-associated shifts in expression that influence pathways central to neuronal maintenance and signaling (Che et al. 2014; Danka Mohammed et al. 2016; Munkhzul et al. 2023). For example, increased expression of *miR-204* has been shown to suppress *EphB2* translation and impair synaptic signaling, while reduced levels of miRNAs targeting *APP* and *BACE1* can facilitate accumulation of inflammatory proteins (e.g., amyloids) (Che et al. 2014; Danka Mohammed et al. 2016). Beyond these direct effects on neuronal pathways, miRNAs also mediate interactions between aging, environmental stressors, and physiological state. Experimental studies in rodents

demonstrate that stress exposure (e.g., sepsis) can induce age- and sex-dependent changes in hippocampal miRNA profiles, altering inflammatory and neuroprotective responses (Rani et al. 2022), while hormonal regulation, including estrogen signaling, further shapes miRNA networks across the lifespan (Rao et al. 2013). Consistent with this, miRNA expression is highly responsive to metabolic and environmental conditions, such as smoke exposure in rhesus macaque plasma and ocular tissues (Smit-McBride et al. 2018) reflecting a dynamic interface between external inputs and gene regulation (T. Singh and Yadav 2020). In humans, altered miRNA expression has been implicated in both normal aging and neurodegenerative disease (Basak et al. 2016). Changes in key neuronal miRNAs, including *miR-9*, *miR-124*, and *miR-132*, have been observed in aged and Alzheimer's disease hippocampus (Lukiw 2007; Llorens et al. 2017), while recent work suggests that a relatively small subset of miRNAs may account for a large proportion of regulatory differences associated with aging and exceptional longevity (Alberro et al. 2024). Despite these advances, most insights into miRNA function in brain aging derive from rodent or late-stage human studies, and relatively little is known about how miRNA expression changes across the lifespan in the primate brain.

Rodent models have provided critical insight into the biology of aging (Nadon 2006; Colman 2018), but important differences in lifespan, developmental timing, and brain maturation limit their ability to fully capture the dynamics of primate brain aging (Glazko et al. 2005; Finch and Austad 2012; Chiou et al. 2020). In particular, rodents lack the prolonged periods of juvenile development and extended adult lifespan that characterize most primates, constraining their utility for studying gradual, lifespan-dependent changes in brain function (Yakovlev 1967; Bogin 1999; Ingram et al. 2001; Gogtay et al. 2004; Nadon 2006; Leonard et al. 2007; Tau and Peterson 2010). Nonhuman primates, such as rhesus macaques (*Macaca mulatta*), provide a powerful

system for investigating brain aging due to their close evolutionary relationship to humans and shared features of neurobiology, physiology, and social behavior (Ingram et al. 2001; Roth et al. 2004; Chiou et al. 2020). In the present study, we first describe the composition of small RNAs detectable in the hippocampus of rhesus macaques. We then focus specifically on miRNA expression, applying time-series modeling approaches to identify miRNAs exhibiting both monotonic and nonlinear expression trajectories across the lifespan and integrating these data with matched transcriptomic data to identify candidate miRNA-gene regulatory relationships. Together, this work provides a characterization of the small RNA landscape in the primate hippocampus and offers new insight into the role of post-transcriptional regulation by miRNAs during the aging process.

4.2 Methods

4.2.1 Samples

This study utilized a subset of 48 archived hippocampal tissue samples from a larger cohort of rhesus macaques described previously (T. J. Anderson et al. 2025). Briefly, samples were obtained from 24 female and 24 male individuals aged 3 to 35 years through the Aging Primate Resource at the Oregon National Primate Research Center (ONPRC). This subset was selected to ensure equal sex representation across the full adult lifespan by randomly sampling individuals from both above and below the mean cohort age (16.1 years). This sampling strategy ensures representation across early, mid, and late adulthood, enabling characterization of age-associated miRNA expression dynamics across the complete adult lifespan while accounting for potential sex-related confounds. Full details regarding sample collection, tissue dissection, and storage protocols are provided in (T. J. Anderson et al. 2025).

4.2.2 Small RNA extraction, library preparation and sequencing

Genomic DNA and total RNA were isolated from hippocampal tissue using the Qiagen DNA/RNA Allprep kit, following the manufacturer's protocols. This extraction protocol ensured that all molecular datasets generated were derived from the same individuals. Extractions were performed by the ONPRC Primate Genetics Core in randomized batches of 24 samples per day to prevent clustering by age or sex during processing.

Small RNA libraries were constructed using the Nextflex Small RNA-Seq v4 kit, which includes a size selection step that removes RNA molecules larger than 100 nucleotides, enriching the libraries for small RNAs such as miRNAs. Library preparation and quality control were conducted according to the kit's protocol. Library preparation and sequencing was carried out at the University of Oregon's Genomics and Cell Characterization Core Facility (GC3F) with sequencing being performed on a single lane of an Illumina NovaSeq 6000 S4 flow cell, generating 100 bp paired-end reads and yielding ~40 million reads per sample.

4.2.3 Data preprocessing

The quality of raw sequencing reads was assessed using FastQC (v0.11.5) (Andrews 2010). Adapter trimming and quality filtering were performed using Cutadapt (v2.10) (Martin 2011), targeting the removal of sequencing adapters as well as poly-G and poly-A tails that arose as artifacts of sequencing. Low-quality bases at the read ends (Phred score <20) were also trimmed. Reads shorter than 18 nucleotides or longer than 30 nucleotides after trimming were excluded from further analysis, consistent with the size range expected for mature miRNAs and the small RNA classes captured by our library preparation approach. Trimmed reads were re-evaluated with FastQC to confirm the effectiveness of these cleaning steps. These preprocessed

reads were then used as input for both the broad small RNA landscape characterization and the miRNA-specific differential expression analysis described below. Data preprocessing was done using the University of Oregon's high performance computing cluster, Talapas. Subsequent data and statistical analyses were performed in R version 4.4.2 & using Talapas.

4.2.4 Small RNA landscape characterization

To obtain an initial characterization of the small RNA species present in our dataset, we performed broad small RNA annotation using QuickMIRSeq (v1.0) (S. Zhao et al. 2017). QuickMIRSeq is a pipeline designed for the simultaneous quantification of known small RNA species from multiple RNA classes, including mature miRNAs, miRNA hairpin precursors, other small noncoding RNAs (tRNAs, rRNAs, snoRNAs, snRNAs), and mRNA-derived fragments. Because QuickMIRSeq is optimized for single-end reads, this analysis was performed using the R2 reads only from our paired-end libraries. A species-specific macaque reference database was constructed using the QuickMIRSeq database framework with the macaque-specific Bowtie index, incorporating known *Macaca mulatta* small RNA sequences drawn from multiple sources: tRNA sequences were obtained from the GtRNAdb genome tRNA database (rheMac8 assembly), noncoding RNA sequences were obtained from Ensembl release 115 (Mmul_10 assembly), and mRNA sequences were obtained from the Ensembl release 115 cDNA annotation for *Macaca mulatta*. Noise filtering was enabled to remove low-confidence read mappings, and a zero-count filter was applied to retain only features detected above an average read threshold of 2 counts across at least 40% of samples. This analysis served as a broad survey of the detectable small RNA landscape in our hippocampal dataset, providing compositional information across RNA classes. Given the limitations of single-end-only compatibility and our interest in

leveraging the paired-end structure of our data for higher-confidence miRNA-specific mapping and downstream integration with matched transcriptomic data, all subsequent differential expression analyses were performed using the pipeline described below.

4.2.5 miRNA differential expression analysis

High-quality trimmed reads were aligned to a custom index of mature *Macaca mulatta* miRNA sequences from miRbase (Griffiths-Jones et al. 2006; Kozomara et al. 2019) using STAR (v2.7.3a) (Dobin et al. 2013). Alignment parameters were selected to enforce stringent mapping appropriate for small RNA molecules: spliced alignments were disabled (--alignIntronMax 1) and up to two mismatches per read were permitted (--outFilterMismatchNmax 2). Mapped reads were quantified using featureCounts (Liao et al. 2014), producing a count matrix. To maximize mapping confidence, only uniquely mapped reads were retained for downstream analysis, resulting in ~5% of total reads being carried forward. While this is a conservative threshold, it reflects a deliberate prioritization of high-confidence miRNA alignments given the inherent challenges of mapping short RNA sequences. We used edgeR (Robinson et al. 2010) and limma (Ritchie et al. 2015) to filter out low count miRNAs and normalize prior to differential expression resulting in a count matrix of 820 mature miRNA sequences used in all downstream analyses.

To account for unmeasured sources of technical variation, we performed surrogate variable analysis (SVA) using the sva R package (Leek et al. 2012) on the normalized miRNA count matrix, using a model adjusted for age and sex. From the resulting surrogate variables (SVs), we retained those explaining >2% of total variance in the count matrix that were not significantly associated with age or sex (FDR < 0.05). These SVs (n = 8) were then included

alongside sex as covariates in a linear model fit using limma (Ritchie et al. 2015), from which residuals were extracted to produce a corrected expression matrix with the effects of sex and latent technical variation removed. This residualized matrix was used as input for downstream differential expression analysis.

miRNA expression dynamics across the adult lifespan were modeled using autoregressive integrated moving average (ARIMA) models, similar to previously described approaches (Márquez et al. 2020; Watowich et al. 2022; T. J. Anderson et al. 2025). Briefly, residualized expression values were aligned by sample age and summarized to generate a single value per miRNA at each time point, producing age-ordered time series. ARIMA models were then fit to each miRNA to identify those exhibiting non-random, age-associated temporal structure. miRNAs with stationary profiles or poorly fit models were excluded based on model parameters and residual diagnostics (Ljung–Box test), retaining only those with significant temporal signal. For these selected age-associated miRNAs, fitted values were smoothed using LOESS to generate continuous expression trajectories across the age range.

To identify groups of miRNAs with similar temporal patterns, we clustered the predicted expression trajectories using a correlation-based distance metric (1 - Pearson correlation) and complete-linkage hierarchical clustering. The optimal number of clusters was determined empirically using k-means clustering on a principal component-reduced representation of the data, with the gap statistic used to select the number of clusters according to the first standard error maximum criterion. This optimal k was then applied to the hierarchical clustering to define final miRNA expression clusters.

4.2.6 Identification of miRNA predicted gene targets

Predicted gene targets for age-associated miRNAs were identified using the miRDB database (v6.0) (Y. Chen and Wang 2020), a machine learning-based resource for miRNA target prediction. Rhesus macaque miRNA identifiers were converted to their human homologs to enable compatibility with miRDB annotations. Target predictions were filtered to retain high-confidence interactions (prediction score ≥ 90) consistent with previous approaches (Corset et al. 2026). Resulting target transcripts were then mapped to gene symbols using the org.Hs.eg.db annotation database, and redundant transcript-level mappings were collapsed to gene-level annotations by retaining the highest-confidence score per miRNA-gene pair. The final dataset consisted of high-confidence predicted target genes for each miRNA that were used for downstream analyses.

4.2.7 Ontology analysis

We performed ontology enrichment analysis for all predicted gene targets of age-associated miRNAs using g:Profiler (Raudvere et al. 2019). Enrichment analysis was conducted for Gene Ontology (GO) Biological Processes and KEGG pathways using the default statistical domain restricted to annotated genes. Significance was determined by Benjamini-Hochberg false discovery rate correction (FDR < 0.05). To enhance specificity, the maximum term size was set to 500 with annotation performed using the human reference genome (hg38). This was done to maintain consistency with the human genome annotations used for miRNA target prediction in miRDB.

4.2.8 Integration with matched gene expression data

To identify candidate miRNA-gene regulatory relationships supported by expression data, we integrated our age-associated miRNA profiles with matched gene expression data from the same individuals, generated previously (T. J. Anderson et al. 2025). Because the miRNA and gene expression datasets were generated from different sample sizes (n = 48 and n = 86 respectively) and thus represent different age point distributions, ARIMA fitted values from each dataset were averaged across individuals sharing the same age to produce a common age-indexed matrix for each data type. Analyses were performed using these common age points.

Candidate miRNA-gene pairs were first defined by intersecting the set of high-confidence predicted target genes (miRDB score ≥ 90) for age-associated miRNAs with the set of differentially expressed genes (DEGs) previously identified (T. J. Anderson et al. 2025). For each resulting miRNA-gene pair, we tested whether the two features exhibited an inverse relationship across the shared age axis – consistent with canonical miRNA-mediated repression – by computing Pearson correlations between their age-averaged expression profiles. P-values were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate procedure, and pairs with a significant negative Pearson correlation ($r < 0$, FDR < 0.05) were retained as exhibiting inverse co-expression.

To further prioritize biologically plausible regulatory candidates, we applied a modulation threshold filter to the inverse pairs. For each pair, a linear model was fit regressing gene expression on miRNA expression across the age-averaged profiles. The modulation percentage was defined as the absolute miRNA-attributable change in gene expression – estimated as $|\beta \times \text{range}(\text{miRNA})|$ – expressed as a proportion of the total age-related amplitude of gene expression. Pairs were retained as candidate regulatory pairs if the miRNA could account

for between 10% and 50% of the gene's age-related expression change. The lower bound was chosen to exclude pairs where the miRNA contribution is negligible relative to total expression change; the upper bound was chosen to exclude pairs where the miRNA alone would implausibly account for the majority of age-related variation, consistent with the established view that individual miRNAs typically exert modest rather than dominant effects on target gene expression (Bartel 2018). This multi-step filtering procedure (requiring predicted targeting, significant inverse correlation, and a modulation estimate within a biologically reasonable range) was designed to generate a high-confidence set of candidate miRNA-gene pairs for downstream interpretation.

4.3 Results

4.3.1 Small RNA landscape characterization

To characterize the diversity of small RNA species detectable in the rhesus macaque hippocampus, we first performed broad small RNA annotation using QuickMIRSeq. Across all 48 samples, QuickMIRSeq detected a total of 901 unique mature miRNA species and 610 unique miRNA hairpin precursors, confirming miRNA representation in the dataset. Beyond miRNAs, additional small RNA classes were detected, including 1,988 unique snRNA features, 1,547 unique snoRNA features, 406 unique tRNA features (including mitochondrial tRNAs), and 315 unique rRNA features (including mitochondrial rRNAs) (**Fig. 4.2** and **Table S4.1**). Among non-miRNA mapped reads, snRNAs accounted for 46.8% of the total, snoRNAs for 36.3%, tRNAs for 9.5%, and rRNAs for 7.4%.

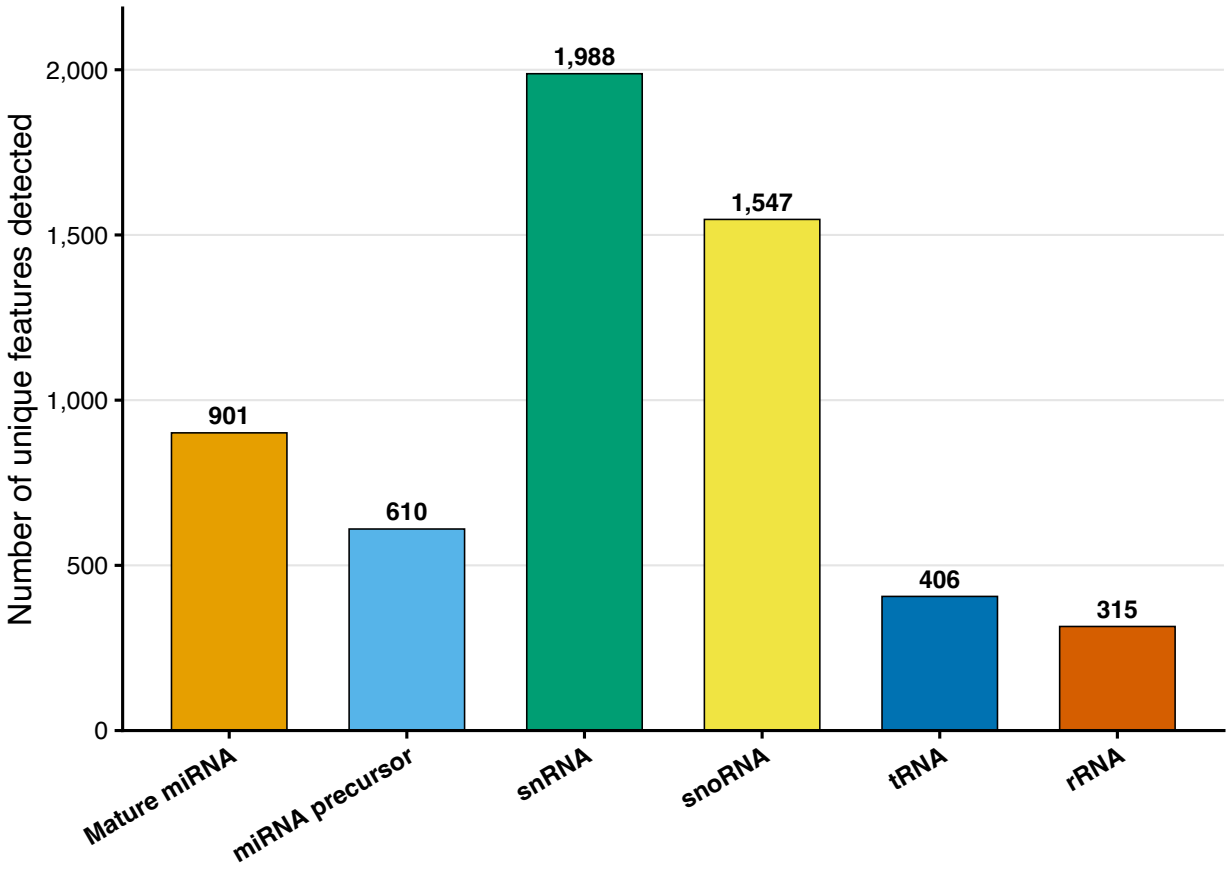


Fig. 4.2 Breakdown of unique small RNA features detected in the rhesus macaque hippocampus. Small RNA feature types are denoted by colors with counts reflecting the number of unique annotated sequences detected

4.3.2 Differential miRNA expression patterns highlight midlife transitions

To enable high-confidence quantification of mature miRNA expression and facilitate integration with matched transcriptomic data (T. J. Anderson et al. 2025), we generated a dedicated count matrix of 820 mature miRNA sequences which formed the basis for all downstream differential expression analyses. First, to assess global patterns of miRNA expression across the cohort, we performed principal component analysis (PCA) on normalized expression values. Samples showed partial separation along the first principal component (PC1), which explained 11.7% of the total variance (**Fig. 4.3A**). Notably, PC1 was significantly associated with age ($\beta = 0.388$, 95% CI [0.084, 0.692], $p = 0.016$) (**Fig. 4.3B**), indicating that age contributes to miRNA expression variation in the hippocampus.

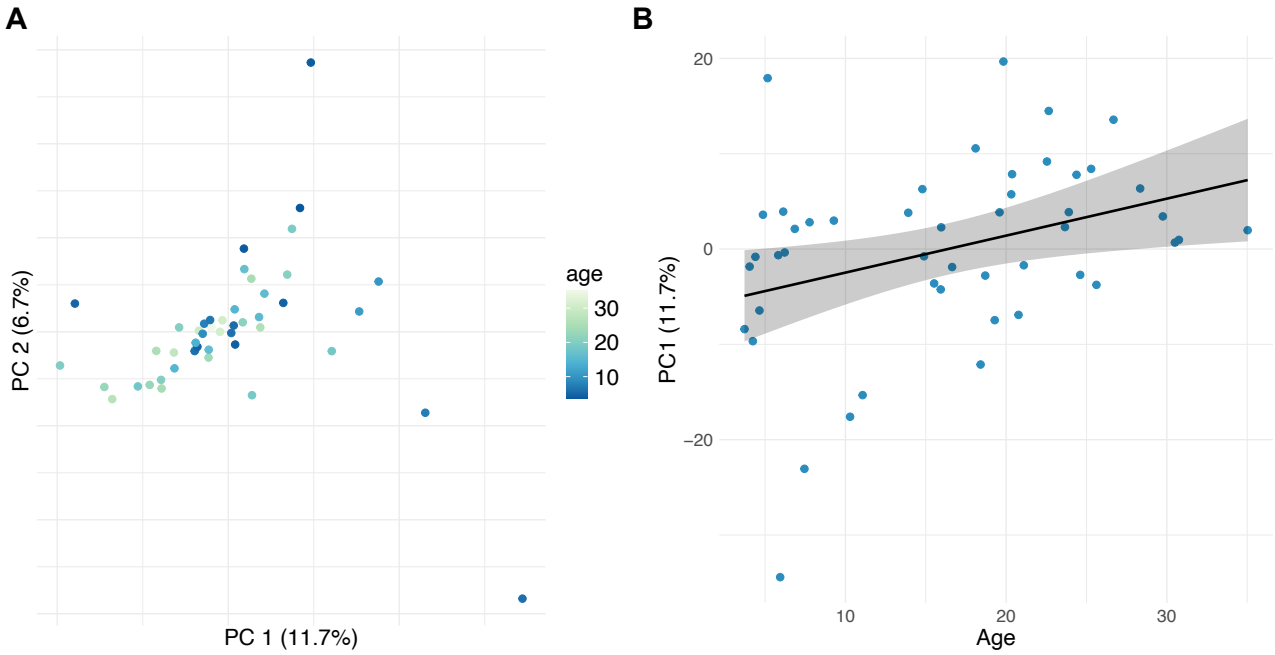


Fig. 4.3 PCA analysis of mature miRNAs. A) PCA of all mature miRNAs from 48 rhesus macaque hippocampus samples denoted by relative ages. B) PC1 was significantly associated with age (β = 0.388, 95% CI [0.084, 0.692], p = 0.016)

To further characterize age-associated miRNA dynamics in the macaque hippocampus, we applied ARIMA-based time series modeling to normalized expression profiles. This analysis identified 91 (11.1% of miRNAs tested) age-associated miRNAs which fit 5 broad patterns of temporal expression as determined by hierarchical and k-means clustering (**Fig. 4.4A** and **Table S4.2**). Two clusters (Cluster 1 [11 miRNAs; 12.1%] and Cluster 2 [40 miRNAs; 43.9%]) showed monotonic trends in expression meaning that miRNAs either consistently increased or decreased in expression across the lifespan (**Fig. 4.4B** and **C** respectively). Meanwhile, three clusters (Cluster 3 [16 miRNAs; 17.6%], Cluster 4 [6 miRNAs; 6.6%] and Cluster 5 [18 miRNAs; 19.8%]) exhibited nonlinear trajectories in expression (**Fig. 4.4 D-F** respectively). To enable functional interpretation, predicted gene targets were identified for age-associated miRNAs. Of the 91 miRNAs, 59 had at least one high-confidence predicted target (miRDB score ≥ 90) and

were retained for downstream enrichment analyses (range: 1-426 predicted targets per miRNA; mean = 85 predicted targets; median = 43 predicted targets).

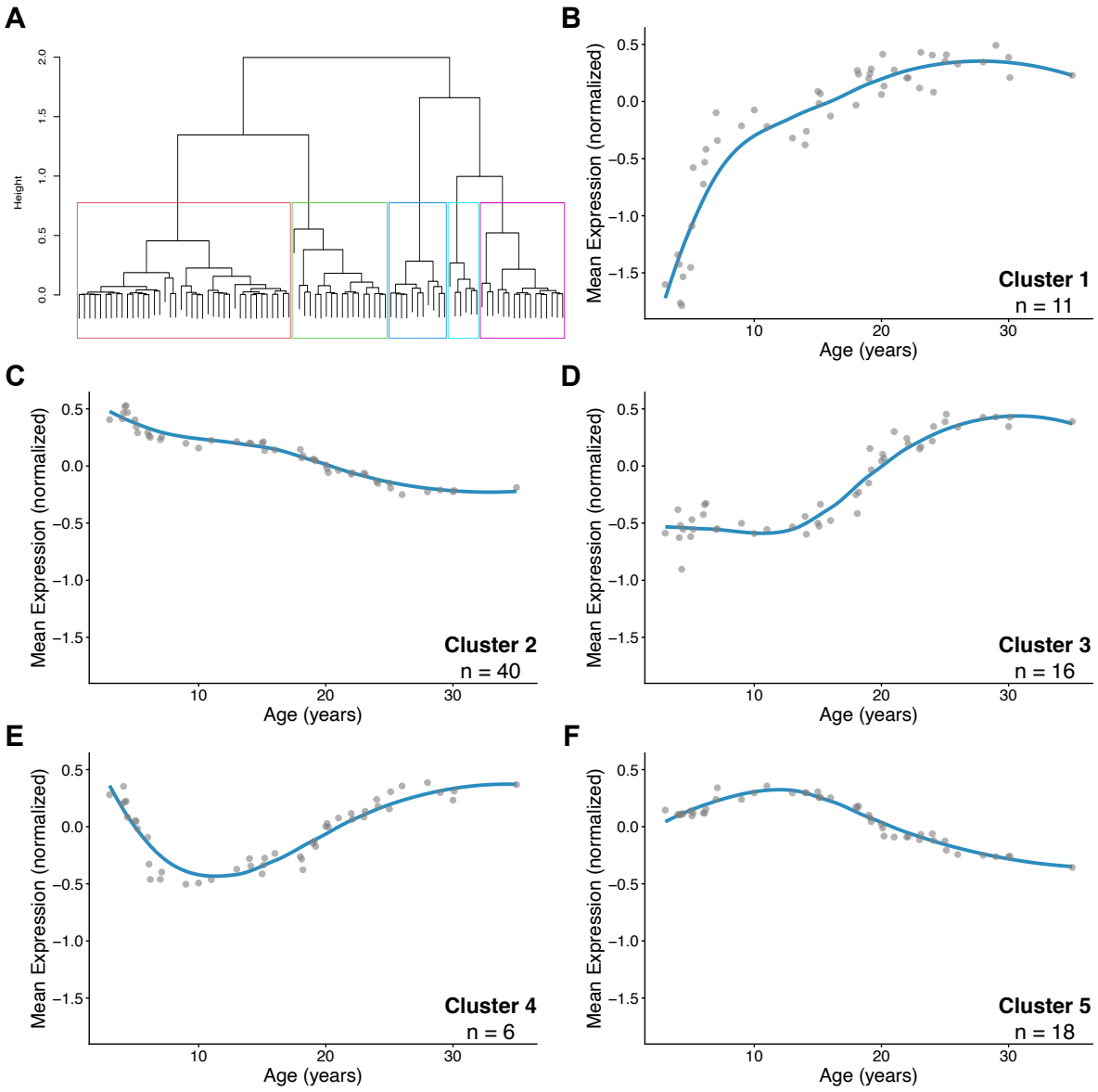


Fig. 4.4 Expression trajectories for miRNAs which fit 5 broad expression patterns. A) Cluster dendrogram of all age-associated miRNAs as identified by ARIMA modeling when controlling for sex. Clusters utilized in analysis are denoted by colored boxes. B-F) Mean predicted expression counts for all miRNAs in each cluster, as determined by ARIMA models. The blue line (LOESS-smoothed curve) represents the average trajectory. Number of genes in each cluster is denoted by n

Cluster 1 contained 11 miRNAs (12.1% of age-associated miRNAs) which typically increased in expression across the adult lifespan (**Fig. 4.4B**) meaning that predicted targets likely

decreased in expression across the lifespan. The top enriched biological processes among predicted gene targets of age-associated miRNAs in this cluster were extracellular matrix organization (GO:0030198), extracellular structure organization (GO:0043062) and external encapsulating structure organization (GO:0045229); top enriched KEGG pathways included protein digestion and absorption (KEGG:04974), MAPK signaling pathway (KEGG:04010) and relaxin signaling pathway (KEGG:04926) (**Table S4.3**).

Cluster 2 contained 40 miRNAs (43.9% of age-associated miRNAs) which typically decreased in expression across the adult lifespan (**Fig. 4.4C**) meaning that predicted targets likely increased in expression across the lifespan. The top enriched biological processes among predicted gene targets of age-associated miRNAs in this cluster were axonogenesis (GO:0007409), regulation of synapse structure or activity (GO:0050803) and regulation of synapse organization (GO:0050807); top enriched KEGG pathways included circadian entrainment (KEGG:04713), axon guidance (KEGG:04360) and parathyroid hormone synthesis, secretion and action (KEGG:04928) (**Table S4.3**).

Cluster 3 contained 16 miRNAs (17.6% of age-associated miRNAs) which exhibited a nonlinear trajectory in expression across the adult lifespan (**Fig. 4.4D**). These miRNAs exhibited relatively stable expression patterns reaching their lowest point at 10.7 years (as estimated by the LOESS-smoothed curve) before increasing in expression trajectory, meaning that predicted targets likely decreased in expression later in life. The top enriched biological processes among predicted gene targets of age-associated miRNAs in this cluster were mitotic cell cycle phase transition (GO:0044772), G1/S transition of mitotic cell cycle (GO:0000082) and regulation of neurogenesis (GO:0050767); top enriched KEGG pathways included axon guidance

(KEGG:04360), cAMP signaling pathway (KEGG:04024) and relaxin signaling pathway (KEGG:04926) (**Table S4.3**).

Cluster 4 contained 6 miRNAs (6.6% of age-associated miRNAs) which exhibited a nonlinear trajectory in expression across the adult lifespan (**Fig. 4.4E**). These miRNAs decreased in expression before reaching their lowest point at 11.3 years (as estimated by the LOESS-smoothed curve) before increasing in expression trajectory, meaning that predicted targets likely exhibited inverse patterns in expression. The top enriched biological processes among predicted gene targets of age-associated miRNAs in this cluster were regulation of angiogenesis (GO:0045765), regulation of vasculature development (GO:1901342) and rhythmic process (GO:0048511); there were no significantly enriched KEGG pathways for predicted gene targets in this cluster.

Cluster 5 contained 18 miRNAs (19.8% of age-associated miRNAs) which exhibited a nonlinear trajectory in expression across the adult lifespan (**Fig. 4.4F**). These miRNAs increased in expression before reaching their highest point at 12 years (as estimated by the LOESS-smoothed curve) before decreasing in expression trajectory, meaning that predicted targets likely exhibited inverse patterns in expression. The top enriched biological processes among predicted gene targets of age-associated miRNAs in this cluster were peptidyl-serine modification (GO:0018209), peptidyl-serine phosphorylation (GO:0018105) and axonogenesis (GO:0007409); there were no significantly enriched KEGG pathways for predicted gene targets in this cluster.

4.3.3 Integration with matched transcriptome data identifies candidate miRNA-gene regulatory pairs

To assess the potential functional relevance of our age-associated miRNAs on influencing transcription, we intersected our list of predicted gene targets with differentially expressed genes (DEGs) identified in our previously described transcriptome analysis (T. J. Anderson et al. 2025). Of the 3,429 unique predicted target genes across the 59 age-associated miRNAs which had predicted targets that passed our confidence threshold, 641 (18.7%) overlapped with the 2,679 DEGs from the matched transcriptome dataset, yielding a total of 963 miRNA-gene pairs across 56 unique miRNAs and 641 unique genes for downstream correlation testing (range of predicted DEG targets per miRNA: 1-86; mean = 17; median = 10).

For each candidate pair, we evaluated whether miRNA and gene expression exhibited an inverse relationship across age – consistent with canonical miRNA-mediated repression – by computing Pearson correlations between their age-averaged ARIMA fitted expression profiles across shared age points. Of the 963 pairs tested, 403 (41.8%) showed a significant negative correlation (Pearson's $r < 0$, FDR < 0.05), spanning 46 unique miRNAs and 327 unique genes (range of significant inverse targets per miRNA: 1-65; mean = 9; median = 4; median Pearson r = -0.678).

To further prioritize biologically plausible regulatory relationships, we applied a modulation threshold filter requiring that the miRNA could plausibly account for between 10% and 50% of the gene's total age-related expression change. This filter yielded 95 high-confidence candidate miRNA-gene regulatory pairs across 29 unique miRNAs and 86 unique genes (range of candidate targets per miRNA: 1-13; mean = 3; median = 2) (**Fig. 4.5**), with a median Pearson r of -0.517 and a median modulation estimate of 42.5%. The progressive narrowing from 963

initial pairs to 95 final candidates is the result of our stringent multi-step filtering approach and aim to retain only the most well-supported miRNA-gene pairs. These candidate pairs represent a prioritized set of putative post-transcriptional regulatory relationships in the aging macaque hippocampus that warrant functional validation in future work (**Table S4.4**).

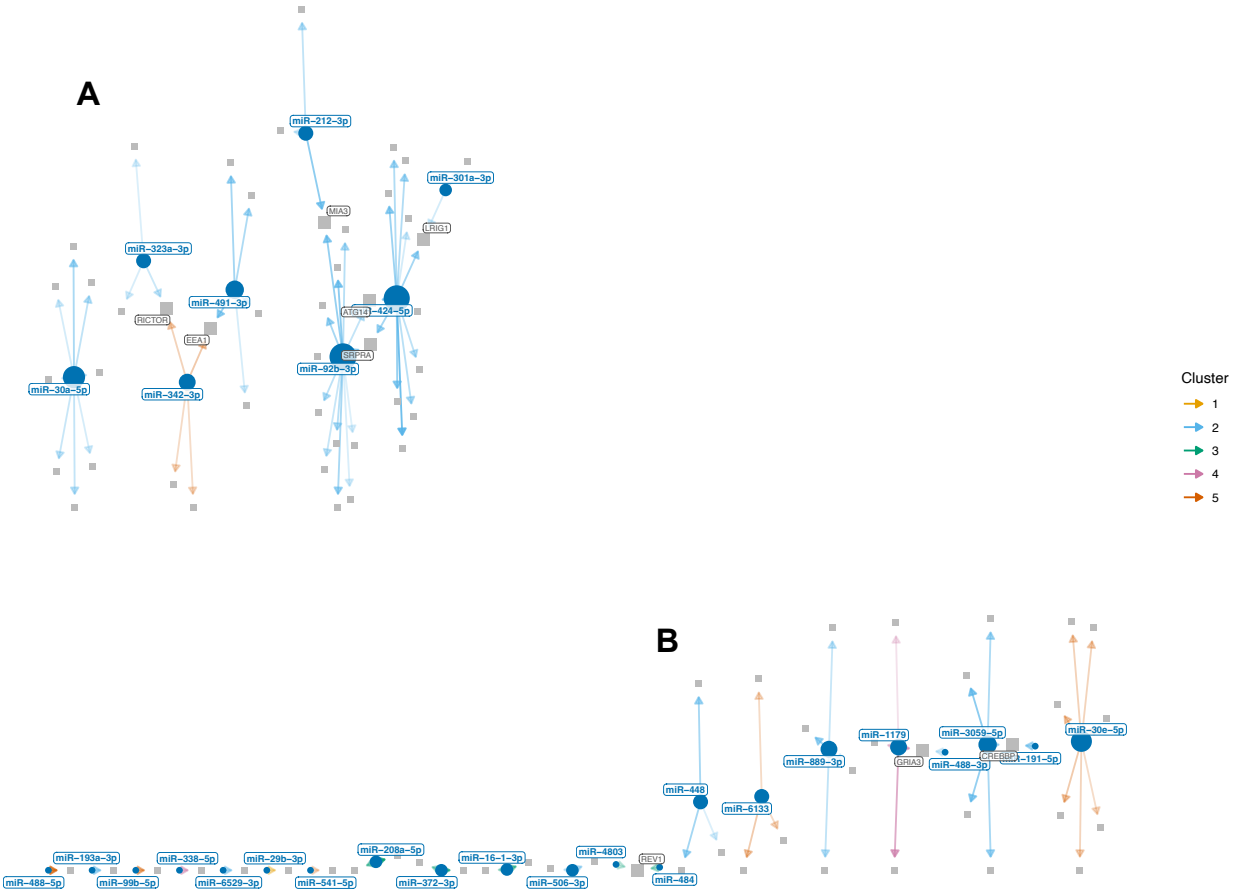


Fig. 4.5 High-confidence miRNA-gene regulatory network. A-B) miRNA-gene regulatory network reveals two spatially distinct miRNA groups with no shared gene targets. Blue circles represent miRNAs (sized by number of connections), gray squares represent target genes. Edges are colored by expression cluster. Gene labels are shown for targets regulated by 2+ miRNAs. Node size reflects the number of high-confidence targets per miRNA, with miR-424-5p and miR-92b-3p emerging as the primary regulatory hubs in this network. Edge length reflects network tension, with shorter edges indicating tightly co-embedded miRNA-gene pairs and longer edges indicating more peripherally connected targets

4.3.4 Sex-specific miRNA expression dynamics

To explore potential sex differences in age-associated miRNA expression, we repeated the ARIMA-based modeling separately for females (n = 24) and males (n = 24). Given that these

analyses represent a further subsetting of an already modest cohort, the results should be interpreted with caution as reduced sample sizes likely limit statistical power to detect age-associated temporal signal. With this caveat in mind, the sex-stratified analyses revealed notable differences in the number and composition of age-associated miRNAs between sexes. In males, 91 age-associated miRNAs were identified fitting 6 broad expression clusters (**Table S4.5**). These clusters included both monotonic trajectories – with the largest cluster (Cluster 1; n = 38) decreasing across the lifespan and cluster 2 (n = 14) increasing – and nonlinear trajectories (Clusters 3-6) highlighting similar midlife transitions as observed in the original approach. In females, 24 age-associated miRNAs were detected, fitting 5 expression clusters (**Table S4.5**), with Cluster 2 being the largest (n = 10) and exhibiting a decreasing trajectory across the lifespan; all other clusters exhibited nonlinear trajectories highlighting similar midlife transitions as observed in the original approach. Overlap between the sex-specific analyses and the original approach was modest and overlap between the two sex-specific sets was minimal, suggesting sex-specific patterns of age-associated miRNA expression are present and should be investigated further (see **Supplemental Results** for full details).

While the reduced power inherent to the sex-stratified analyses may contribute to the lower number of age-associated miRNAs detected in females, the minimal overlap between male and female age-associated miRNA sets suggests the possibility of sex-specific patterns of miRNA expression dynamics in the aging primate hippocampus that warrant further investigation in larger cohorts.

4.4 Discussion

To better understand how post-transcriptional regulation by miRNAs may contribute to brain aging, we characterized age-associated miRNA expression in the hippocampus of rhesus macaques. Using time-series modeling, we identified miRNAs exhibiting both monotonic and nonlinear age-associated expression trajectories, with nonlinear clusters converging on transitions centered just after ~10 years of age in macaques (roughly ~30 years in humans) (**Fig. 4.4**). Through integration with matched transcriptomic data from the same individuals, we identified candidate miRNA-gene regulatory pairs with relevance to brain aging and neurodegenerative disease susceptibility. Together, these findings extend our understanding of post-transcriptional regulation in the aging primate brain and provide a foundation for future mechanistic investigation.

In this study we identified age-associated miRNAs with expression trajectories that change direction during midlife (**Fig. 4.4 D-F**). We previously identified analogous midlife transitions at the transcriptional level and have discussed hypotheses regarding their molecular basis (T. J. Anderson et al. 2025). The inflection points observed here correspond to known changes in white matter volume in both macaques and humans (S. Wang and Young 2014; Cox et al. 2016; Jiskoot et al. 2018; Reas et al. 2018; Kubicki et al. 2019) and may also reflect the completion of brain maturation (Arain et al. 2013); these interpretations are not mutually exclusive and may reflect a regulatory reorganization. Ontology results from the nonlinear clusters support this interpretation. The enrichment of axon guidance, regulation of neurogenesis, and axonogenesis among predicted targets in Clusters 3 and 5 – of which *NCAM1*, *NFIB* and *MAP6* are gene targets in our high-confidence target list – is consistent with maturation-related processes transitioning during midlife (**Table S4.3**). This interpretation is further supported by

the presence of predicted targets directly implicated in myelination and oligodendrocyte differentiation – including *MYRF* and *QKI* in Cluster 3, *TARDBP* and *MAP6* in Cluster 4, and *NRXN1* and *SPAST* in Cluster 5 – suggesting that the miRNA expression transitions we observe may reflect coordinated post-transcriptional regulation of white matter maturation and maintenance processes across the primate adult lifespan.

The directionality of age-associated miRNA expression provides important biological context for interpreting these findings. The largest group of age-associated miRNAs decreased in expression across adulthood (Cluster 2; n = 40). This pattern is consistent with a progressive reduction in post-transcriptional regulatory precision with age, reflecting a broader molecular dysregulation that has been documented across multiple levels of gene regulation in the aging brain (Nativio et al. 2018; López-Otín et al. 2023). Importantly, the directional relationship between a miRNA and its target gene has functional implications worth considering. When a miRNA decreases in expression, its target genes are expected to increase – meaning that the age-associated loss of these miRNAs may contribute to the upregulation of genes they normally suppress. Future work examining the specific functional identities of target genes in this context will be important for understanding the full consequences of this regulatory shift.

Several of our pathway-level findings are further reinforced by our candidate miRNA-gene regulatory pairs, where genes driving the enrichment of axonogenesis (e.g., *SMURF1*, *PTPRM*, *MAP1B*, *NCAM1*, *NFIB*, and *MAP6*) were also retained as high-confidence regulatory targets, suggesting that miRNA-mediated regulation of axonal and synaptic integrity may be meaningfully altered across the primate adult lifespan. Among the miRNAs exhibiting this decreasing trajectory, several have direct relevance to brain aging and neurodegeneration in humans. Notably, the human ortholog of mml-miR-9-3p, which we found decreases across the

lifespan in the macaque hippocampus, is differentially expressed in both normal aged brains and individuals with AD (Lukiw 2007), raising questions about its potential role in primate hippocampal aging more broadly. Cluster 5 miRNAs (n = 18) also decrease later in life and include predicted targets enriched for axonogenesis, further highlighting this pathway as potentially relevant to primate hippocampal aging. However, unlike those in Cluster 2, miRNAs in this cluster first increase in expression before declining after a peak around ~12 years, suggesting that miRNA-mediated regulation of axonal processes may be altered at different timepoints in the aging trajectory. Whether this represents a delayed protective response that eventually wanes, or an early indicator of later-life vulnerability in axonal maintenance pathways, remains an open question worth exploring through functional validation.

A smaller group of age-associated miRNAs increased in expression with age. Cluster 1 (n = 11) showed monotonically increasing expression across the lifespan, with predicted gene targets enriched for extracellular matrix organization and MAPK signaling. The progressive upregulation of miRNAs targeting these pathways is consistent with age-associated disruption of intercellular communication, a recognized hallmark of aging (López-Otín et al. 2023), as both extracellular matrix remodeling and MAPK signaling are central to the maintenance of cellular microenvironments and the propagation of signals between cells in the brain.

Cluster 3 (n = 16) exhibited a distinct and biologically interesting pattern – relatively stable expression across early and mid-adulthood before increasing in expression after ~10.7 years – meaning predicted targets are likely suppressed later in life. The top enriched processes among Cluster 3 predicted targets included regulation of neurogenesis and mitotic cell cycle transitions, which is notable given that the hippocampus is one of the few brain regions where neurogenesis persists into adulthood (Eriksson et al. 1998; Disouky et al. 2026). The late-life

upregulation of miRNAs targeting neurogenesis-associated genes may therefore reflect a post-midlife transition in the regulation of adult hippocampal neurogenesis. Whether this shift represents a response to changing hippocampal demands or a contributor to age-related decline warrants further investigation.

Cluster 4 (n = 6) – miRNAs decrease in expression in early in adulthood before increasing in late adulthood (>11.3 years). This cluster was notably enriched for regulation of angiogenesis and vasculature development, raising the possibility that the post-midlife increase in these miRNAs could contribute to age-associated reductions in cerebrovascular function. This is particularly relevant given the well-established role of angiogenic dysregulation in shaping neurodegenerative disease susceptibility (Kieliszek-Ryba et al. 2026), and the fact that the hippocampus is among the first brain regions to exhibit blood-brain barrier breakdown with age (Montagne et al. 2015). miRNA-mediated suppression of pro-angiogenic pathways may therefore represent one post-transcriptional mechanism contributing to the heightened vulnerability of the aging hippocampus.

Several of our candidate miRNA-gene regulatory pairs (**Table S4.4**) are implicated in brain aging and neurodegenerative disease susceptibility. Collectively, these pairs represent candidates worth investigating further given their potential roles in shaping primate brain aging trajectories and disease vulnerability and may offer entry points for future aging intervention studies. Below, we highlight a few of interest.

miR-212-3p, which is part of the miR-132/212 cluster and has been shown to be consistently downregulated in AD brains with greater magnitude in gray matter (Pichler et al. 2017), was predicted to modulate expression of SLC6A1, a GABA transporter previously identified as a marker of AD pathology in rodent models (Chum et al. 2024). The finding that

miR-212-3p decreases across the macaque lifespan (Cluster 2) suggests it may play a role in normal primate brain aging more broadly, though the precise relationship between its age-associated decline in healthy individuals and its more pronounced dysregulation in AD remains unclear and may differ across species and disease contexts.

miR-29b-3p, which we identify as an age-associated miRNA with a predicted regulatory relationship to its target gene (*FAM167A*), is among the most consistently upregulated miRNAs during aging across multiple tissues and species, and has been linked to age-related cognitive decline as well as downregulation in neurodegenerative disorders including AD (Hébert et al. 2008). Indeed, we observe similar age-related expression patterns for this miRNA (Cluster 1).

miR-3059-5p was predicted to target *CAVIN1*, a gene encoding a scaffolding protein required for caveolae biogenesis that stabilizes caveolin-1 (CAV1) at the plasma membrane. Caveolin-1 expression in the brain increases with age and is considerably elevated in α-synuclein transgenic mice, with caveolin-1 overexpression facilitating neuronal uptake of α-synuclein and formation of Lewy body-like inclusions – implicating the caveolin-1/CAVIN1 axis in age-related PD susceptibility (Ha et al. 2021). Notably, *CAVIN1* was identified as a DEG that increased in expression across the macaque lifespan in our matched transcriptome analysis (T. J. Anderson et al. 2025), and miR-3059-5p is derived from Cluster 2, the cluster exhibiting monotonic decreases in expression across adulthood. This pattern is consistent with a model in which the age-associated decline of miR-3059-5p progressively releases *CAVIN1* from post-transcriptional repression, contributing to its increased expression in the aging hippocampus.

Finally, miR-1179 also stands out as a promising candidate worth further investigation, as it targets *GRIA2* and *GRIA3* and is implicated in the development of neurodegenerative disease (Abuelezz et al. 2021). These genes encode AMPA receptor subunits GluA2 and GluA3, which

are integral in synaptic transmission and plasticity and serve as links between amyloid-β levels and AD in rodent models (Hettinger et al. 2018). Interestingly, miR-1179 is derived from Cluster 4 (nonlinear expression trajectory), further reinforcing midlife as a potentially critical timepoint in shaping later-life brain aging.

Visualization of the miRNA-gene regulatory network reveals two spatially distinct miRNA groups with no shared gene targets (**Fig. 4.5A** and **4.5B**), indicating they operate in separate regulatory contexts. Within the larger group, the majority of high-confidence miRNA-gene relationships belong to Cluster 2, consistent with the observation that Cluster 2 contributes the largest share of candidate pairs. This aligns with the broader pattern of miRNA downregulation across the lifespan described above, and with evidence from the literature that age-associated loss of miRNA expression is a pervasive feature of brain aging (Nativio et al. 2018; López-Otín et al. 2023; Munkhzul et al. 2023). miR-424-5p and miR-92b-3p emerge as the primary regulatory hubs in this network. Several genes – including *MIA3*, *EEA1*, *ATG14*, *SRPRA*, *LRIG1*, *GRIA3*, *CREBBP*, *RICTOR*, and *REV1* – are targeted by two or more age-associated miRNAs, suggesting convergent post-transcriptional regulation of these genes in the aging hippocampus. The predominance of Cluster 2 relationships in the final candidate set suggests that the progressive decline in miRNA expression across the primate adult lifespan may have downstream consequences, with the decreasing abundance of these miRNAs potentially releasing their target genes from post-transcriptional repression and contributing to increased target gene expression with age – a pattern consistent with a broader breakdown of post-transcriptional regulatory control in the aging hippocampus.

Despite growing interest, the role of small non-coding RNAs in the context of brain aging remains poorly understood. To address this gap, we sought to characterize small non-coding

RNA expression in hippocampus tissue derived from rhesus macaques sampled across adulthood. We found representation for a diverse catalog of small RNAs including both mature and precursor miRNAs, snRNAs, snoRNAs, tRNAs and rRNAs. While not the primary focus of this study, the detected small RNA classes and their relative representation are broadly consistent with patterns reported in small RNA-seq studies of mammalian brain tissue, where miRNAs, snRNAs, and snoRNAs represent the predominant detectable classes (Isakova et al. 2020; Fafard-Couture et al. 2021). The relative representation of each class likely reflects both biological abundance and the current completeness of macaque small RNA annotations, which vary across RNA classes and may limit detection of less well-characterized species. Nevertheless, these findings confirm that our sequencing approach captures a diverse repertoire of small noncoding RNA species across multiple classes in the macaque hippocampus, providing a foundation for future studies examining the full complement of small RNA dynamics across the primate adult lifespan.

Several limitations should be considered when interpreting these findings. This study is cross-sectional, which limits our ability to directly infer within-individual miRNA trajectories across the lifespan. While our modeling approach captures population-level age-associated dynamics, longitudinal sampling (which is not feasible for primate brain tissue) would be necessary to distinguish true within-individual remodeling from population-level heterogeneity. Second, miRNA profiles were generated from bulk hippocampal tissue, meaning that the expression changes we detect likely reflect a combination of cell-intrinsic regulatory remodeling and shifts in cellular composition across age. Future studies integrating single-nucleus small RNA profiling will be critical for resolving the cell-type-specific contributions of candidate miRNAs to hippocampal aging. Third, our analyses are anchored to known *Macaca mulatta*

miRNA sequences catalogued in miRBase, meaning that novel or unannotated miRNAs are not captured in the current dataset. Given that miRBase annotations for nonhuman primates remain incomplete relative to humans, there is likely a subset of functionally relevant miRNAs expressed in the macaque hippocampus that are not represented here; future work incorporating *de novo* miRNA discovery approaches will be important for providing a more complete picture of miRNA dynamics in aging primate brain tissue. Fourth, the candidate miRNA-gene regulatory pairs identified through our integration analysis are based on predicted targeting and correlational evidence, and because candidate pairs were defined by intersecting miRNA targets with genes showing statistically significant differential expression, this approach is inherently biased toward miRNA-gene relationships that manifest as detectable mRNA-level changes. Age-associated miRNAs may exert additional regulatory effects through translational repression without measurable mRNA degradation, or through buffering roles that stabilize rather than significantly alter transcript abundance. The candidates highlighted above are therefore of interest precisely because they survive this stringent filtering, but the full scope of miRNA-mediated regulation in the aging macaque hippocampus is likely broader than what is represented here. Functional validation through direct experimental manipulation will be necessary to establish causal regulatory relationships and to uncover additional miRNA targets not detectable through mRNA-level integration alone. Finally, the relatively modest sample size, while comparable to similar nonhuman primate aging studies, limits statistical power, particularly in the sex-stratified analyses. Despite these limitations, this dataset spans the full adult lifespan of rhesus macaques and is derived entirely from neuropathologically normal individuals, providing a rare and valuable window into the molecular dynamics of healthy primate brain aging.

This study provides one of the first characterizations of the small RNA landscape in the aging primate hippocampus, revealing a diverse repertoire of detectable small RNA classes alongside age-associated miRNA expression dynamics. By demonstrating that miRNA expression is not simply linear but includes nonlinear trajectories that converge on a midlife transition period, this work adds post-transcriptional regulation to the growing body of evidence that midlife is a molecularly significant period in primate brain aging. The candidate miRNA-gene regulatory pairs identified through multi-layer integration with matched transcriptomic data provide a prioritized set of targets for future mechanistic investigation into the post-transcriptional regulation of brain aging and disease susceptibility in primates. Ultimately, by characterizing the small RNA regulatory landscape of the aging primate hippocampus and identifying candidate post-transcriptional regulatory relationships with relevance to human brain aging and disease, this work lays the groundwork for future studies aimed at understanding the molecular mechanisms that shape brain aging trajectories across the primate lifespan.

Bridge to Chapter V

Chapter 4 extended the multi-omic framework of this dissertation to the post-transcriptional level, demonstrating that miRNA expression in the rhesus macaque hippocampus is dynamically remodeled across the adult lifespan encompassing both monotonic trajectories and nonlinear transitions that converge on a midlife inflection point consistent with those identified in Chapters 2 and 3. Through integration with matched transcriptomic data, Chapter 4 further identified candidate miRNA-gene regulatory relationships with relevance to brain aging and neurodegenerative disease susceptibility, adding a post-transcriptional dimension to the regulatory networks shaping hippocampal aging. Together, Chapters 2 through 4 establish that

aging in the primate hippocampus is not confined to any single molecular layer, but involves coordinated remodeling across transcriptional, epigenetic, and post-transcriptional systems with shared hallmarks of aging, evidence for regulatory drift, and a recurring midlife transition detectable across all three layers. Chapter 5 synthesizes these findings within a broader biological and anthropological framework, situating the molecular signatures of hippocampal aging identified here within the context of human neurodegenerative disease susceptibility and the potential for regulatory interventions to modify aging trajectories.

CHAPTER V

CONCLUSION

Aging is a complex biological process characterized by a multitude of molecular and cellular changes across the lifespan (Balcombe and Sinclair 2001; Weinert and Timiras 2003; López-Otín et al. 2023; Metcalf et al. 2026). Importantly, while core mechanisms of aging appear broadly conserved across biological systems, this process is heterogeneous in its timing, pace and manifestation, varying across species, tissues, and individuals (Buffenstein 2009; Lemaître et al. 2013; Brinkmeyer-Langford et al. 2016; Reichard 2016; Roach 2016; Rando and Wyss-Coray 2021; Yamamoto et al. 2022). The brain exemplifies this variability, as different species exhibit distinct trajectories of brain aging (R. Peters 2006; Finch and Austad 2012), certain regions are more vulnerable than others (R. Peters 2006; Anand and Dhikav 2012; Bartsch and Wulff 2015; de Flores et al. 2015), and individuals differ markedly in the rate and extent of age-associated cognitive decline. This heterogeneity is especially pronounced in humans, where some individuals develop neurodegenerative diseases such as Alzheimer's disease, while others maintain cognitive function well into old age (Langa 2018; Patel et al. 2022; Disouky et al. 2026). Despite this variability, the molecular mechanisms that contribute to differential aging trajectories, particularly in the brain, remain incompletely understood. A major barrier has been the limited availability of human and nonhuman primate brain tissue spanning the full lifespan, which has constrained our ability to characterize how the brain changes at the molecular level with age and to identify the regulatory mechanisms that may underlie variation in aging trajectories. Integrative, multi-layered analyses of gene regulatory systems are particularly valuable in this regard, as they have the potential to capture how molecular systems

interact and respond to both intrinsic programs and environmental signals across the lifespan providing insight into the sources of variation in aging outcomes that single-layer approaches cannot. This dissertation addresses these gaps by integrating transcriptional, epigenetic, and post-transcriptional (miRNA) data across the lifespan in the hippocampus – a brain region particularly susceptible to aging and implicated in neurodegenerative disease – of rhesus macaques, a nonhuman primate demonstrated to be a tractable model of human aging (Chiou et al. 2020).

This dissertation demonstrates that aging in the hippocampus is not characterized by isolated changes within individual molecular layers, but by coordinated remodeling of gene regulatory systems across the lifespan. Across transcriptomic, epigenetic, and post-transcriptional (miRNA) datasets, I identify consistent directional shifts, including coordinated midlife transitions, and candidate cross-layer regulatory associations that together suggest an interconnected molecular response to aging. While functional validation of these candidate relationships will be required, they highlight potential mechanisms through which regulatory remodeling at one layer may shape gene expression and overall aging trajectories.

The molecular changes identified across this dissertation map onto several core hallmarks of aging, providing multi-layer grounding for processes previously characterized largely at the cellular or organismal level. At the epigenetic level, age-associated increases in CpG-level methylation variance and coordinated region-level methylation changes are consistent with epigenetic alterations, one of the primary hallmarks, reflecting the progressive erosion of epigenetic precision that characterizes aging across tissues and species (Seale et al. 2022; López-Otín et al. 2023). Notably, DNAm regions exhibiting nonlinear trajectories are enriched for processes related to intercellular communication, implicating altered intercellular communication as a hallmark that is detectable in the epigenetic landscape of the aging primate hippocampus. At

the transcriptional level, age-associated upregulation of inflammatory pathways is consistent with the hallmark of chronic inflammation, while downregulation of metabolic and mitochondrial gene networks reflects mitochondrial dysfunction – antagonistic and integrative hallmarks that are well established features of brain aging across mammals (C. K. Lee et al. 2000; T. Lu et al. 2004; López-Otín et al. 2023; T. J. Anderson et al. 2025). At the post-transcriptional level, age-associated miRNAs with predicted gene targets enriched for cell signaling pathways further implicate altered intercellular communication as a recurrent theme across regulatory levels. Collectively, these findings demonstrate that the hallmarks of aging are not only detectable across distinct molecular layers but converge on shared biological processes, suggesting that coordinated disruption of intercellular communication, inflammatory regulation, and metabolic homeostasis may be particularly central to hippocampal aging in primates.

Beyond the hallmarks framework, the findings of this dissertation also speak directly to the concept of regulatory drift: the progressive erosion of molecular precision that has been proposed as a unifying mechanism linking age-associated molecular dysfunction to cellular and tissue-level decline (Y. Li and Tollefsbol 2016; Oh et al. 2016; Signor and Nuzhdin 2018; López-Otín et al. 2023). Evidence for regulatory drift is apparent across all three molecular layers examined here. At the epigenetic level, the observation that the vast majority of age-associated CpGs increase in methylation variance with age, and that highly methylated or unmethylated sites in early adulthood converge toward intermediate methylation levels over time, is consistent with a progressive loss of epigenetic fidelity. Furthermore, the predominance of age-associated methylation loss at the region level may render regulatory elements increasingly accessible to transcriptional machinery in ways that promote aberrant expression patterns. At the transcriptional level, older individuals (>20 years) show markedly increased

variance in gene expression relative to younger adults, with the majority of genes exhibiting age-associated increases in expression variability (T. J. Anderson et al. 2025) – supporting the view that aging is accompanied by a breakdown in the precision of transcriptional control. At the post-transcriptional level, the observation that the majority of age-associated miRNAs decrease in expression across the lifespan is consistent with a degradation of post-transcriptional regulatory capacity, potentially allowing transcripts to escape normal miRNA-mediated control. Together, these multi-layer patterns converge on a picture in which hippocampal aging is characterized not only by directional shifts in molecular state, but by a broad reduction in regulatory precision lending empirical support for regulatory drift being a dominant feature in aging and suggesting that this process operates across interconnected layers of gene regulation rather than within any single molecular system.

A central aim of this dissertation was to define candidate gene regulatory networks involved in hippocampal aging – moving beyond the characterization of individual molecular layers toward an integrated understanding of how regulatory systems interact across the lifespan. This goal is addressed in part through the integration of DNAm and miRNA data with matched transcriptomic profiles from the same individuals, enabling the generation of hypotheses about how epigenetic and post-transcriptional changes may interact to shape age-associated gene expression patterns. These analyses, discussed in detail in Chapters 3 and 4, identify candidate cross-layer regulatory relationships. For example, instances where age-associated methylation changes in gene bodies or at regulatory regions that correspond to transcriptional shifts in associated genes, and instances where age-associated miRNA expression changes predict alterations in the abundance of their target transcripts. Together, these represent candidate mechanisms through which regulatory remodeling at one layer may shape gene expression.

While these networks remain to be functionally validated, they provide a framework for prioritizing targets for future experimental work and highlight specific points of regulatory convergence that may be particularly relevant to hippocampal aging. Future work that explicitly models the joint contributions of epigenetic, transcriptional, and post-transcriptional changes within a unified network framework will be essential for determining how these interactions collectively shape aging trajectories and, ultimately, whether disruption of specific regulatory nodes contributes to differential vulnerability to cognitive decline and neurodegeneration.

An important empirical contribution of this dissertation is the generation of a multi-omic dataset from matched hippocampal tissue sampled from both male and female rhesus macaques across adulthood. Primate brain samples with this breadth of age coverage, particularly those including aged individuals, are scarce, making this a valuable resource for the field. These data will be made publicly available, providing a foundation that other researchers can leverage to investigate molecular aging in the primate brain. Importantly, the integration of matched transcriptomic, epigenetic, and post-transcriptional data from the same individuals enables direct investigation of cross-layer regulatory relationships, rather than inference drawn across independent datasets – a study design that provides considerably more power to identify meaningful associations within a unified biological context.

A key insight emerging from this dissertation is that these regulatory changes do not occur linearly but also unfold across distinct phases of the lifespan. Across all three molecular layers, I identified coordinated shifts centered around ~10 years of age in macaques (corresponding to ~30 years in humans) marking a midlife inflection point that may be integral in shaping the trajectory of primate brain aging. This finding provides molecular grounding for a body of epidemiological and neuroimaging evidence suggesting that midlife represents a critical

and underappreciated window for brain health, during which vascular risk factors, lifestyle exposures, and structural brain changes begin to accumulate in ways that predict late-life cognitive outcomes and neurodegenerative disease risk (Raz et al. 2010; Debette et al. 2011; Tolppanen et al. 2012; Hatton et al. 2018; Kubicki et al. 2019; Schubert et al. 2019). While these observations have previously implicated midlife as consequential for long-term brain health, the underlying molecular mechanisms have remained poorly defined. The coordinated regulatory transitions identified here – spanning epigenetic, transcriptional, and post-transcriptional layers – suggest that midlife may represent a period during which gene regulatory systems undergo reorganization, rather than simply reflecting the passive accumulation of damage over time. These findings therefore reframe midlife not merely as a risk exposure window, but as a phase of active molecular transition with potentially lasting consequences for regulatory stability and brain health.

Collectively, these findings offer new insight into how brain aging unfolds at the molecular level in a large-brained, longer-lived primate. A critical feature of the rhesus macaque as a model system is that, despite sharing many of the molecular hallmarks of aging characterized here, these animals do not develop the full neuropathological profile of human neurodegenerative diseases such as Alzheimer's disease. This distinction means that the regulatory changes identified in this dissertation represent signatures of normative primate brain aging rather than disease-driven dysregulation, providing a molecular baseline that remains poorly characterized. By establishing what gene regulatory aging looks like in a nonhuman primate, this work creates a reference framework against which human trajectories can be compared. Although directly testing how these regulatory changes contribute to disease susceptibility was beyond the scope of this dissertation, the identification of coordinated midlife

transitions, candidate cross-layer regulatory networks (many implicated in neurodegenerative diseases) and hallmark-associated molecular changes in a system that does not progress to neurodegeneration offers a powerful foundation for future comparative work. Determining where and how human regulatory aging deviates from these primate patterns will be essential for understanding why our species is disproportionately vulnerable to neurodegeneration.

5.1 Outstanding questions and future directions

The findings of this dissertation should be interpreted in light of several methodological considerations that also point toward important future directions. First, all molecular datasets were generated from bulk hippocampal tissue, which captures global patterns of regulatory aging but does not resolve cell-type-specific dynamics. The observed molecular changes likely reflect a combination of cell-intrinsic regulatory remodeling and age-related shifts in cellular composition, and while cell-type deconvolution analyses were performed on the RNA-seq data to estimate cellular contributions, these approaches are inference-based. Future work integrating single-nucleus or single-cell multi-omic approaches will be essential for disentangling these contributions and determining whether the coordinated regulatory shifts identified here are driven by specific cell populations or reflect broad tissue-level remodeling. Second, the cross-sectional design of this study, while necessary given the practical constraints of primate brain tissue collection, limits inference about within-individual aging trajectories and the causal nature of observed transitions. Longitudinal sampling would be required to determine whether the midlife transitions identified across molecular layers reflect true within-individual regulatory reorganization or population-level heterogeneity – a distinction that will be important for understanding whether these transitions represent a universal feature of primate brain aging or

are shaped by individual life history. Third, samples were obtained from a tissue archive and collected across an extended period of approximately 25 years as part of independent studies, introducing potential heterogeneity from environmental variation, prior interventions, and unmeasured exposures that cannot be fully accounted for. While this variability reflects real-world biological diversity and all individuals were neuropathologically normal, future studies with more tightly controlled sampling designs will strengthen causal inference. Collectively, these considerations highlight that while this dataset represents a rare and valuable resource for studying molecular brain aging in a long-lived primate, the findings presented here are best understood as a foundation for future mechanistic and comparative work rather than a definitive account of the regulatory architecture of hippocampal aging.

Beyond these methodological considerations, several broader biological questions emerge from this work that will be critical for advancing our understanding of primate brain aging and its relevance to human health.

One key question is the extent to which the molecular aging patterns identified in the hippocampus are conserved across brain regions. While the hippocampus is particularly vulnerable to aging and neurodegeneration, other regions, such as the prefrontal cortex (PFC), play equally critical roles in higher-order cognition and brain regions have been demonstrated to exhibit distinct transcriptional identities (Chiou et al. 2023). Ongoing work in the Sterner Lab, including the dissertation research of Megan McNamara, is beginning to address this question by characterizing lifespan gene expression dynamics in the macaque PFC using a comparable dataset. Preliminary findings suggest that midlife transitions are also present in the PFC, although the midlife inflection point is less pronounced than in the hippocampus. This raises the possibility that these transitions reflect a broader, brain-wide reorganization of regulatory

systems during aging, while also highlighting region-specific differences in the timing and magnitude of these changes.

A related question is how environmental and population-level factors shape these molecular aging trajectories. The animals included in this study were derived from a captive research population, raising the possibility that observed patterns may be influenced by environmental conditions, diet, or life history. Comparative analyses in other populations, such as the semi-free-ranging rhesus macaques of Cayo Santiago, Puerto Rico, offer an opportunity to test the robustness and generalizability of these findings. Although shorter lifespans in these populations may limit the ability to assess late-life changes, they provide a critical framework for understanding how ecological and social environments become biologically embedded across the lifespan. Expanding these analyses across multiple primate populations (e.g., Oregon National Primate Research Center, Wisconsin National Primate Research Center, and Caribbean Primate Research Center Cayo Santiago Field Station) will be essential for disentangling conserved aging mechanisms from environmentally contingent effects.

Another important direction is understanding the mechanisms that underlie resilience versus vulnerability to neurodegeneration. Unlike humans, rhesus macaques rarely develop severe neurodegenerative pathology despite exhibiting many of the same molecular hallmarks of aging (Walker and Jucker 2017). Previous work suggests that macaques maintain expression of genes related to synaptic plasticity into older ages compared to humans (Y. Zhu et al. 2018), which may contribute to their relative cognitive resilience. Future work should aim to determine whether this reflects enhanced regulatory maintenance or differences in how age-associated dysregulation propagates across molecular layers. Integrating comparative datasets across

species, particularly in matched brain regions, will be critical for identifying the regulatory features that distinguish healthy aging from disease susceptibility.

A key priority for future work is the functional validation of the candidate cross-layer regulatory relationships identified in this dissertation. While the integration of DNAm, transcriptomic, and miRNA data from matched individuals provides a powerful framework for generating hypotheses about how regulatory layers interact during aging, these associations remain correlative. Determining whether age-associated methylation changes at specific regulatory regions causally influence transcriptional output, or whether the decline of particular miRNAs directly contributes to the dysregulation of their predicted target transcripts, will require targeted experimental approaches. These could include *in vitro* manipulation of candidate miRNA expression in hippocampal cell models, CRISPR-based editing of candidate regulatory regions, or the use of organoid systems that recapitulate aspects of primate brain aging. Prioritizing candidates that are implicated across multiple molecular layers, that map onto hallmark-associated pathways such as intercellular communication and inflammatory regulation or are implicated in neurodegenerative disease pathways will be especially valuable for identifying regulatory nodes with the greatest potential relevance to hippocampal aging and neurodegeneration.

An additional direction that warrants dedicated future investigation is the role of biological sex in shaping molecular aging trajectories in the primate brain. While sex was not the primary focus of this dissertation, preliminary analyses presented in the data chapters suggest that male and female macaques may exhibit differences in the magnitude, timing, or molecular character of age-associated regulatory changes. This is consistent with a growing body of evidence demonstrating sex-specific patterns of epigenetic and transcriptional aging across

tissues and species, including in the brain (Masser et al., 2017; Chinn et al., 2021). Given well-documented sex differences in the risk, onset, and progression of neurodegenerative diseases such as Alzheimer's disease in humans, understanding how biological sex modulates molecular aging in the primate hippocampus is an important and underexplored question. Future work should build explicitly on the preliminary findings presented here by designing analyses with sufficient statistical power to characterize sex-specific aging trajectories across all three molecular layers, and by identifying differences between males and females in the timing and composition of regulatory changes.

Collectively, these future directions highlight the importance of integrating comparative, environmental, and experimental approaches to move beyond descriptive models of aging and toward a mechanistic understanding of how gene regulatory systems shape lifespan trajectories. Addressing these questions will be critical for determining not only how aging unfolds in the primate brain, but also why humans may be uniquely susceptible to neurodegenerative disease.

5.2 Modifying biological aging trajectories: epigenetic clock development and intervention applications

A central question emerging from this work is whether biological aging trajectories can be altered or disrupted, and if so, through what regulatory mechanisms. Epigenetic clocks provide a powerful framework for addressing this question by enabling quantification of biological age and its deviation from chronological age. As part of this dissertation, I developed a hippocampus-specific epigenetic clock that demonstrated high accuracy in predicting chronological age across 86 individuals, with a strong correlation between predicted and actual age (Pearson’s r = 0.93, 95% CI: 0.89–0.95, p = 2.2e-16) and a median absolute error (MAE) of

2.22 years (**Fig. 5.1**) using a modified approach derived from (Goldman et al. 2022; Sadoughi et al. 2025) (See **Supplemental Methods** for detailed description of clock model construction).

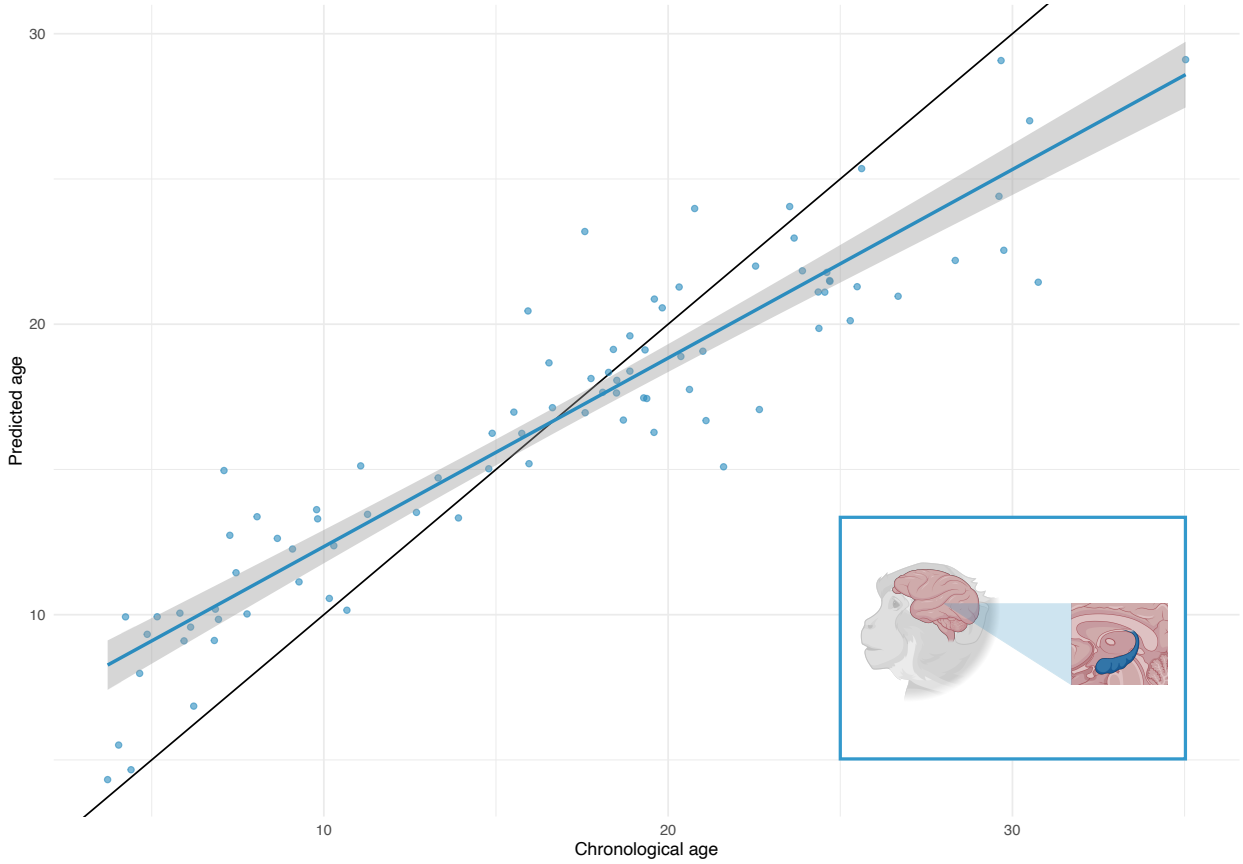


Fig. 5.1 Hippocampus-specific epigenetic clock predicts age using LOOCV. Linear regression of predicted age onto chronological age. Black line is x=y and colored regression line is best linear fit. Generated using 86 rhesus macaque hippocampus samples (r = 0.93, 95% CI: 0.89–0.95, p = 2.2e-16; MAE = 2.22 years)

As a proof-of-concept application, our clock was used to evaluate the effects of rapamycin, a well-established anti-aging intervention with known geroprotective effects that acts as an inhibitor of the mTOR pathway (Benjamin et al. 2011; Wilkinson et al. 2012; Bitto et al. 2016; Towner et al. 2021). Banked hippocampal tissue from 12 individuals (8 rapamycin-treated, 4 controls) was provided by collaborator Dr. Mary Zelinski at OHSU, collected as part of an independent study. While the clock accurately predicted chronological age in control individuals (r = 0.94, 95% CI [−0.23, 0.99], p = 0.062; MAE = 3.11), it was less effective in rapamycin-

treated individuals (r = 0.50, 95% CI [−0.31, 0.89], p = 0.205; MAE = 4.21). This may be expected given the role of rapamycin as an anti-aging intervention. A t-test comparing residual epigenetic age between groups revealed no significant difference (t = 1.30, df = 9.73, 95% CI [−2.07, 7.79], p = 0.225), however there was a trend toward age deceleration in the rapamycin-treated group, with an average reduction of 2.86 years in epigenetic age. The observed effect size for the difference in residual epigenetic age between groups was medium (Cohen's d = 0.60), and power analysis indicated that approximately 22 participants per group would be required to detect this effect with 80% power at α = 0.05 (Torchiano 2016; Champely et al. 2020).

In parallel, differential methylation analysis between rapamycin-treated and control individuals revealed genome-wide methylation gains in treated individuals that contrast with the pattern of progressive methylation loss observed across normal hippocampal aging in this dissertation – a pattern potentially consistent with a partial rescuing effect of rapamycin on the aging methylome. Enrichment analyses of differentially methylated CpGs implicated pathways relevant to brain aging and function, including calcium signaling, dopaminergic synapse regulation, and circadian entrainment among sites losing methylation, and regulation of RNA metabolic processes and axon guidance among sites gaining methylation (full results described in **Supplemental Results**). Together, these findings suggest that rapamycin treatment is associated with methylation remodeling at sites implicated in brain function, even in the absence of a statistically significant shift in epigenetic age.

While effects of rapamycin on lifespan and even brain function have been studied in rodents (D. Guo et al. 2013; Zeng et al. 2008; Wesseling et al. 2017; Erlich et al. 2007), little is known about the effects of rapamycin on the aging brain epigenome, especially in primates. The general trend toward methylation gains observed in rapamycin-treated individuals is notable in

the context of this dissertation, as progressive methylation loss at both the CpG and region level was identified as a consistent feature of normal hippocampal epigenetic aging in macaques. This opposing directionality raises the possibility that rapamycin may partially counteract age-related methylation loss at a genome-wide level. Consistent with this interpretation, studies examining caloric restriction in rhesus macaques have similarly reported methylation gains in treated individuals (Kim et al. 2016; Hadad et al. 2018), potentially suggesting a "rescuing" effect of these anti-aging interventions. Future work explicitly testing the overlap between intervention-associated and age-associated methylation changes will be important for determining whether these effects reflect targeted reversal of epigenetic aging or a more general shift in the methylome.

Complementary work examining caloric restriction provides an opportunity to extend these questions beyond the brain and into systemic aging processes. In collaboration with Dr. Kirstin Sterner and Dr. Lucia Carbone, I am involved in an NIH-funded R21 examining how caloric restriction influences epigenetic aging in the liver of rhesus macaques. Using RRBS-derived DNA methylation data from 96 individuals, we have developed a liver-specific epigenetic clock that accurately predicts chronological age and are currently applying this model to independent cohorts from the NIA and Wisconsin National Primate Research Center, each of which includes both control and calorie-restricted individuals. This work enables direct testing of whether caloric restriction is associated with epigenetic age deceleration and provides a framework for comparing intervention effects across tissues.

Together, these intervention-based approaches raise broader questions about whether conserved anti-aging strategies act through shared regulatory pathways or exhibit tissue-specific

effects, and whether modulation of molecular aging trajectories can meaningfully alter long-term health outcomes.

5.3 Closing remarks

The findings of this dissertation support a shift in how we conceptualize brain aging – from a process driven primarily by the accumulation of molecular damage to one characterized by coordinated changes in gene regulatory systems (López-Otín et al. 2023). Aging in the hippocampus is not confined to a single molecular layer, nor can it be explained by isolated disruptions. Instead, it reflects a systems-level process involving both structured remodeling and stochastic variation across transcriptional, epigenetic, and post-transcriptional levels. These interconnected changes are consistent with a growing body of evidence suggesting that aging emerges, in part, from a progressive loss of regulatory coordination in which interactions among molecular layers become increasingly destabilized over time (Y. Li and Tollefsbol 2016; Oh et al. 2016; Seale et al. 2022; López-Otín et al. 2023), and the multi-layer patterns identified here lend empirical support to this framework in the context of the primate brain.

Within this framework, midlife emerges as a critical phase of brain aging. Rather than viewing aging solely through the lens of late-life decline or disease onset, the results presented here highlight midlife transitions as key inflection points that may shape downstream trajectories. Coordinated shifts across molecular layers around ~10 years of age in macaques (corresponding to ~30 years in humans) suggest that the foundations of later-life brain health may be established decades before the onset of overt pathology. This is consistent with recent multi-omic studies in humans demonstrating that aging is characterized by nonlinear molecular transitions rather than gradual linear change, with distinct periods of accelerated reorganization

across the lifespan (Shen et al. 2024; X. Ma et al. 2026). Together, these findings emphasize the importance of a lifespan perspective in which the timing and dynamics of regulatory change, not just their magnitude, play a central role in determining aging outcomes.

These findings also provide insight into the evolutionary biology of aging. The conservation of core molecular hallmarks across rhesus macaques and humans is consistent with shared primate aging mechanisms, but this work goes a step further by beginning to identify the specific genes, pathways, and regulatory interactions that may underlie meaningful variation between species. The identification of candidate regulatory networks – including those implicated in intercellular communication, inflammatory regulation, and synaptic function – provides a foundation for comparative analyses aimed at determining where human regulatory aging diverges from the primate baseline and whether these divergences contribute to our disproportionate vulnerability to neurodegenerative disease.

From a translational perspective, these results underscore the importance of identifying when and how aging trajectories can be modified. The convergence of molecular changes at midlife highlights a potential window during which interventions may be most effective, while the development of epigenetic clocks provides a quantitative framework for measuring biological aging and evaluating intervention efficacy. The candidate regulatory networks and hallmark-associated pathways identified here also offer molecular targets for future intervention studies, providing a path toward earlier detection of age-related dysregulation and more precise targeting of interventions aimed at promoting brain health and reducing neurodegenerative disease risk.

Ultimately, this work demonstrates that understanding brain aging requires an integrative, systems-level approach that considers how gene regulatory processes change across the lifespan. By leveraging a nonhuman primate with close relevance to humans, this dissertation provides a

framework for linking molecular dynamics to broader aging trajectories in the brain. In doing so, it offers insight into where humans may diverge from other primates and how shifts in the regulation, timing, and stability of gene regulatory systems may contribute to our heightened vulnerability to neurodegenerative disease.

APPENDIX A

SUPPLEMENTAL FIGURES

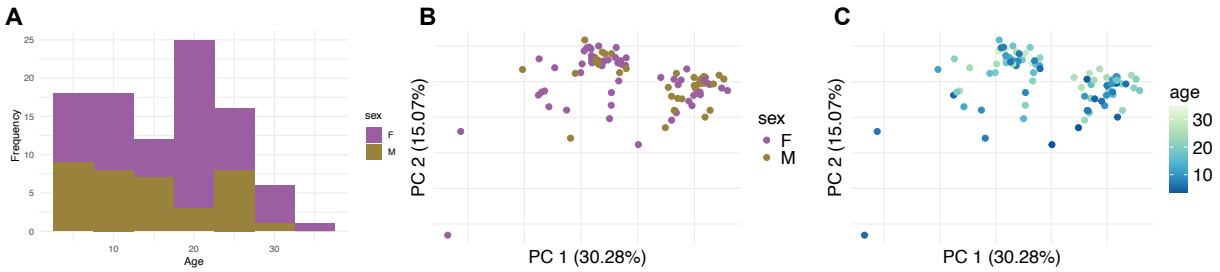


Fig. S2.1 Sample distribution and PCA analysis of 96 hippocampus samples. A) Distribution of ages for 96 hippocampus samples denoted by sex. B) PCA analysis of 96 hippocampus samples. Individuals denoted by sex, C) Individuals denoted by age (PC1 = 30.28%; PC2 = 15.07%)

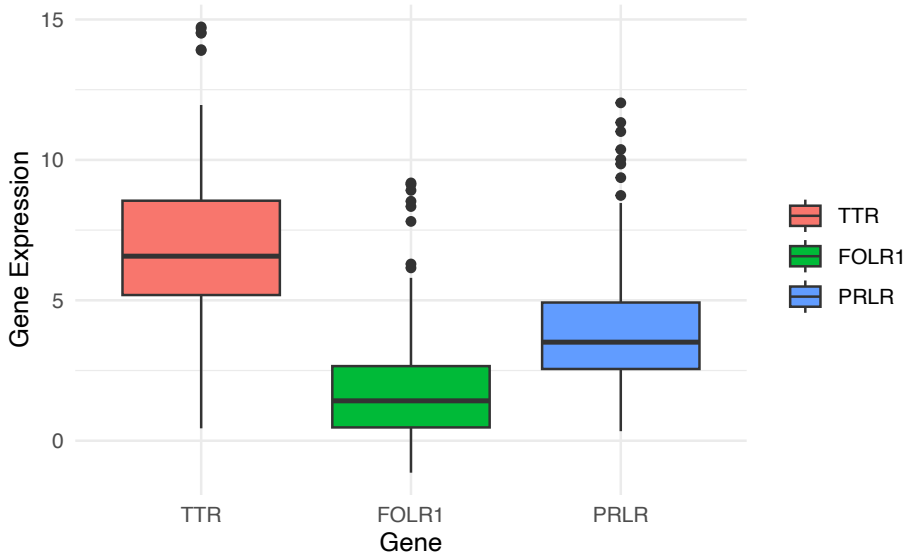


Fig. S2.2 Normalized expression plots of three known choroid plexus marker genes

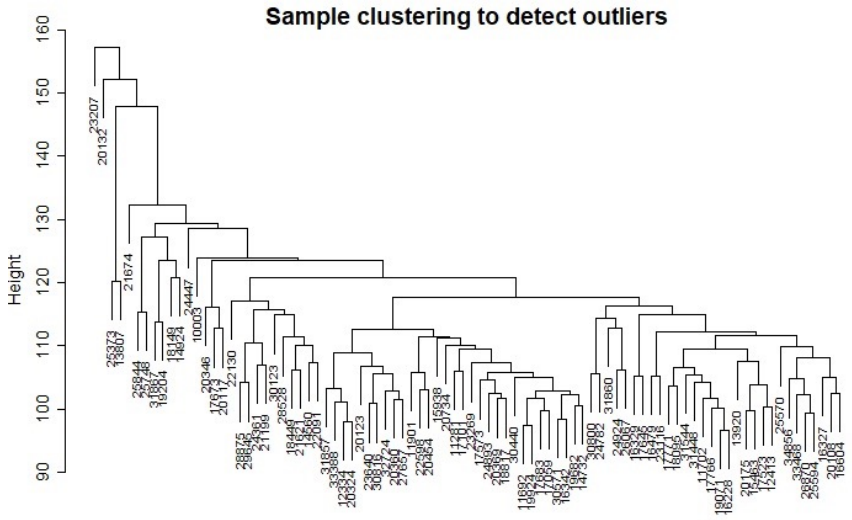


Fig. S2.3 Hierarchical sample clustering analysis using a distance matrix to detect outliers. Individual IDs 23207 and 20132 were the most separated by the analysis

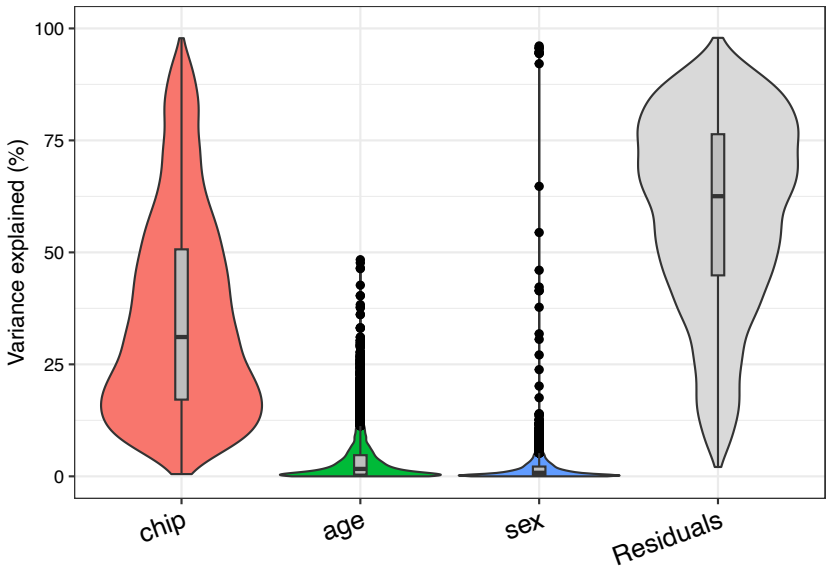


Fig. S2.4 Variance partitioning analysis of gene expression data (formula = gene expression ~ age + sex + chip)

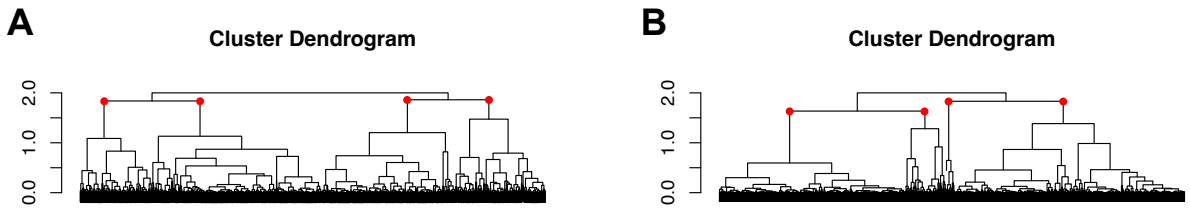


Fig. S2.5 Hierarchical clustering analysis shows 4 broad expression patterns for female and male DEGs. A) Cluster Dendrogram of all significantly differentially expressed genes as identified by ARIMA modeling for females and B) males respectively. Clusters utilized in analysis are denoted by red nodes

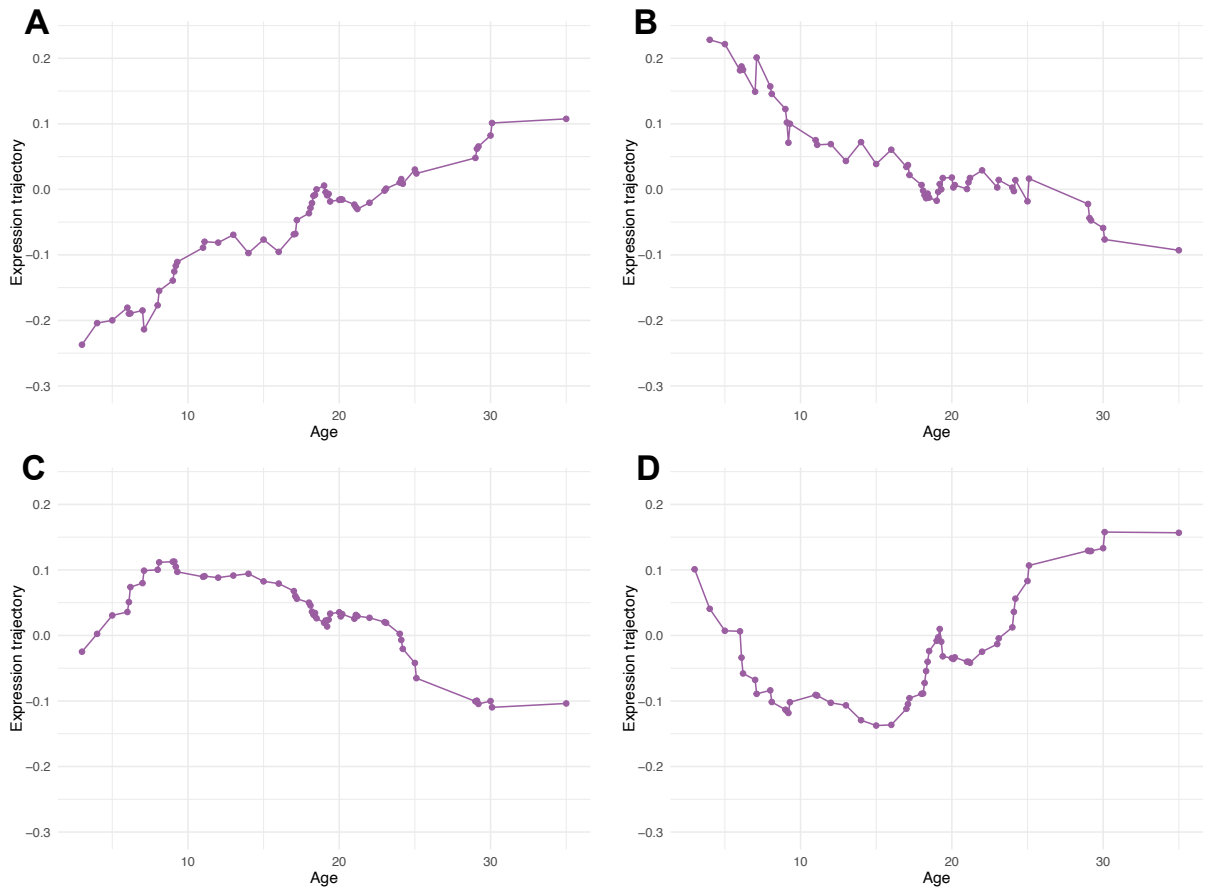


Fig. S2.6 Expression trajectories of female gene clusters. A) Averaged expression trajectory for all genes (n=757) in Cluster 1 (upregulated across age), B) all genes (n=593) in Cluster 2 (downregulated across age), C) all genes (n=400) in Cluster 3 (first nonlinear age-associated cluster) D) and for all genes (n=318) in Cluster 4 (second nonlinear age-associated cluster)

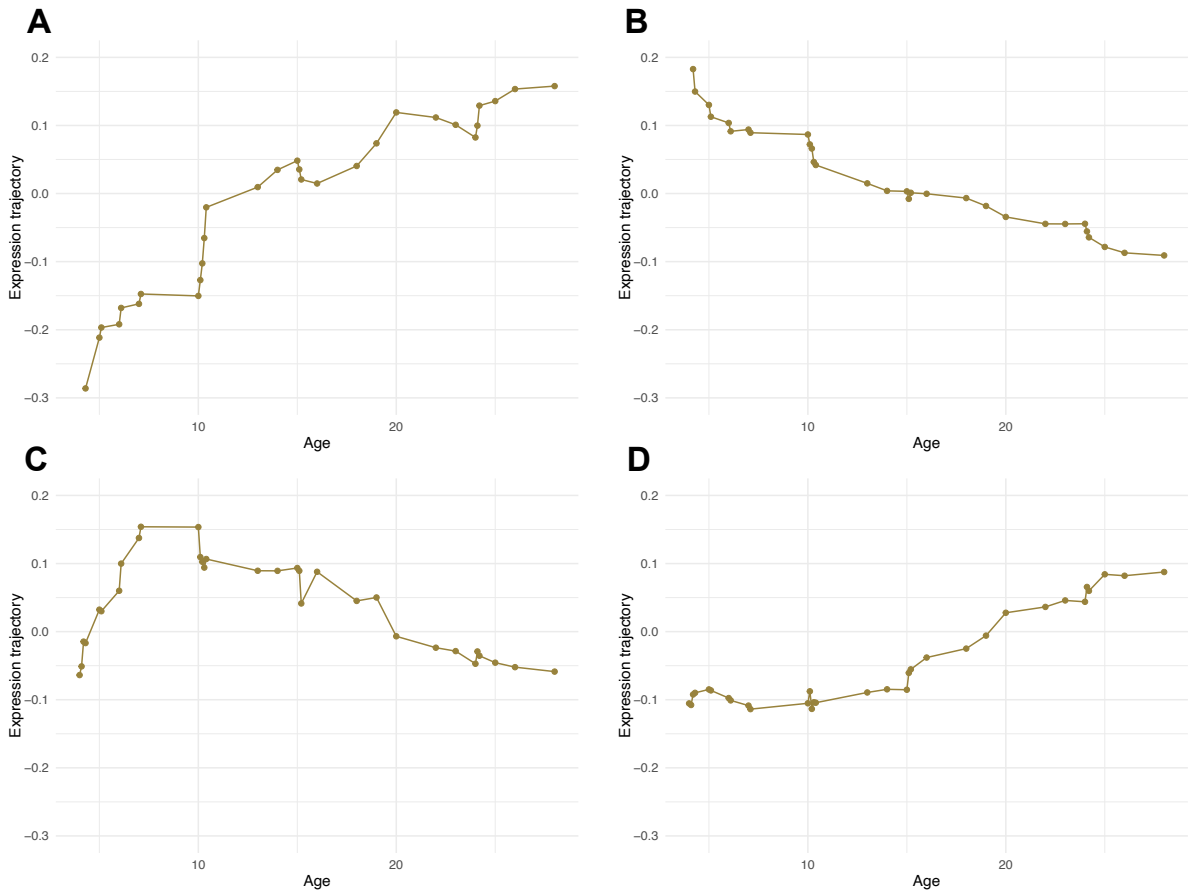


Fig. S2.7 Expression trajectories of male gene clusters. A) Averaged expression trajectory for all genes (n=698) in Cluster 1 (upregulated across age), B) all genes (n=861) in Cluster 2 (downregulated across age), C) all genes (n=31) in Cluster 3 (nonlinear age-associated cluster) D) and for all genes (n=152) in Cluster 4 (age-associated cluster)

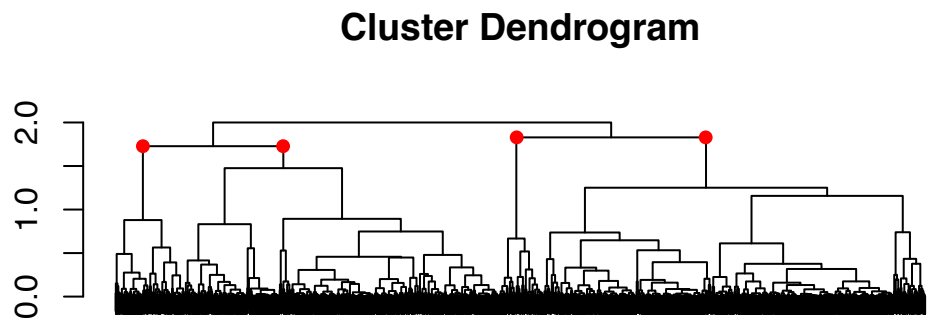


Fig. S2.8 Cluster Dendrogram of all significantly differentially expressed genes as identified by ARIMA modeling when controlling for cell-type proportion changes. Clusters utilized in analysis were denoted by red nodes

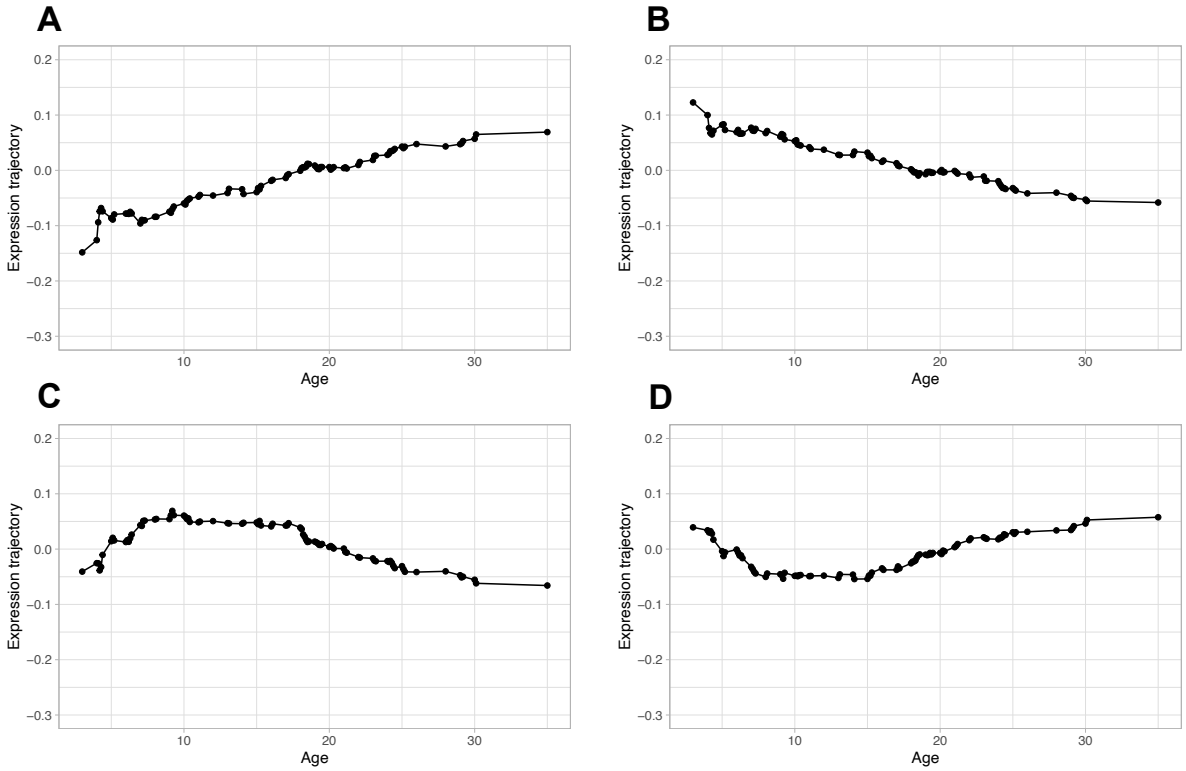


Fig. S2.9 Averaged expression trajectories for genes in each distinct cluster when controlling for cell-type proportion changes. A) Cluster 1 contains 1,026 genes largely upregulated across age. B) Cluster 2 contains 845 genes largely downregulated across age. C & D) Clusters 3 and 4 contained 188 and 118 genes respectively and both exhibit nonlinear trends in expression

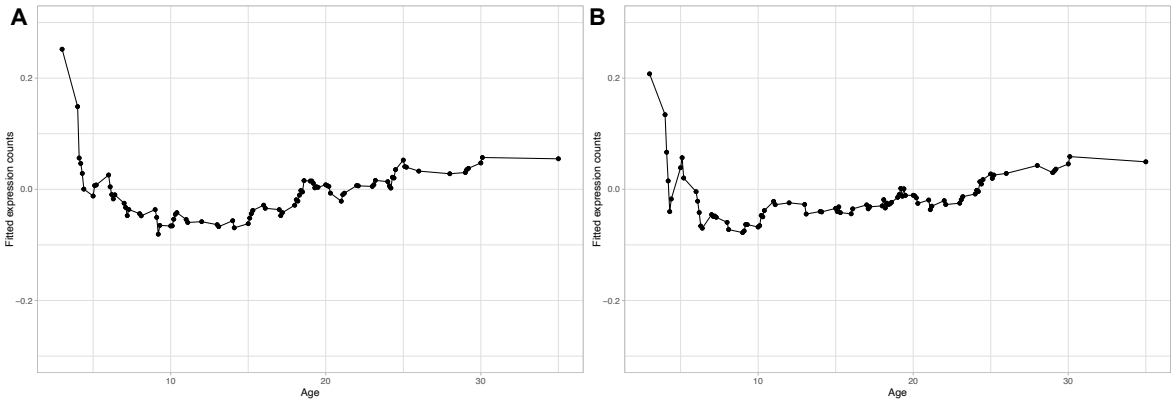


Fig. S2.10 Fitted expression trajectories over the lifespan for A) *SIRT1* and B) *SIRT2* respectively. Expression results are from full dataset when controlling for sex

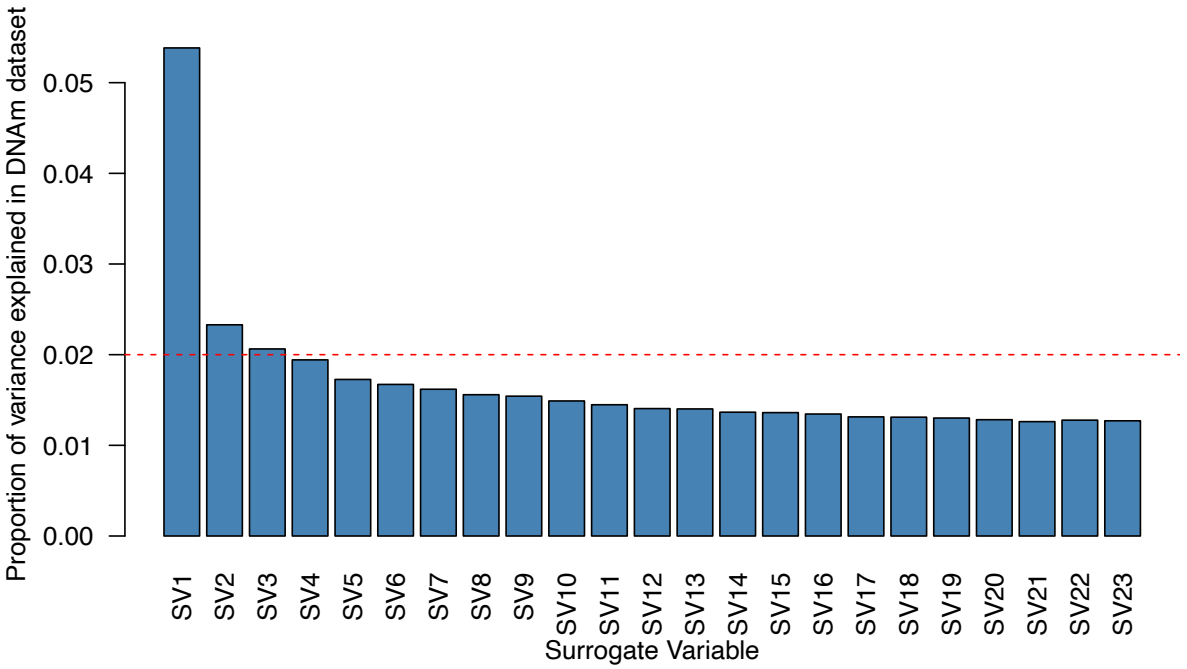


Fig. S3.1 Top 3 SVs included as covariates in downstream analyses. SVs explaining >2% of the total variance in the dataset, but were not significantly associated with sex or age, were included as covariates in downstream analyses to control for potential latent technical effects

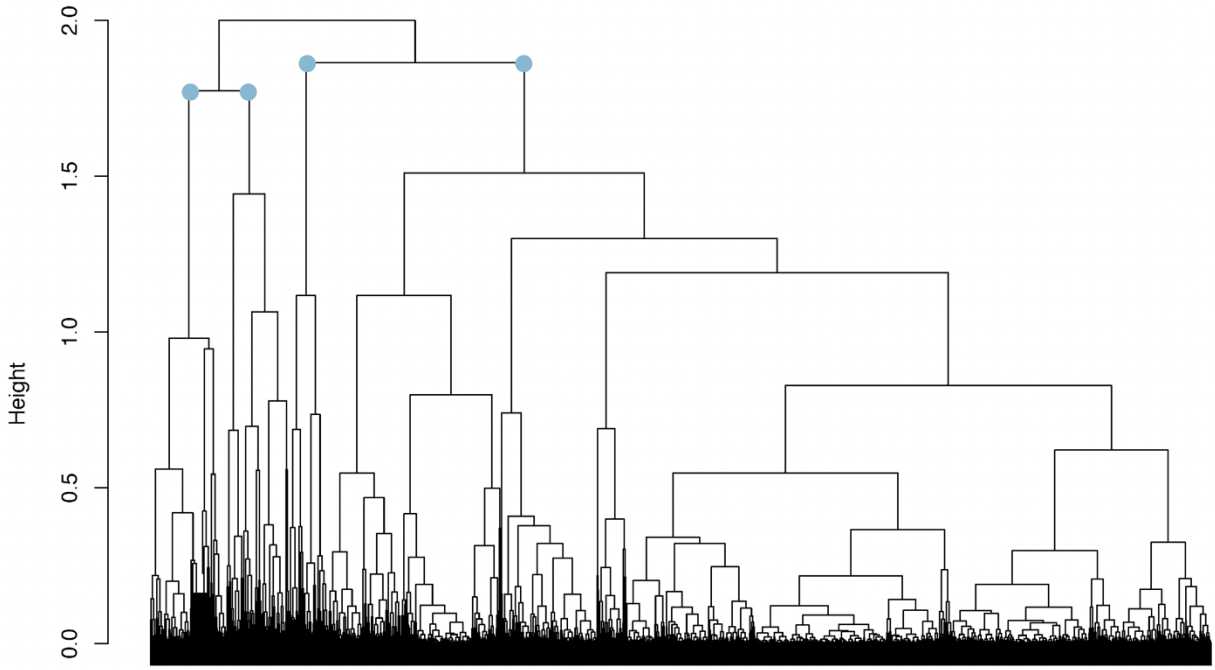


Fig. S3.2 Cluster dendrogram of all age-associated regions as identified by ARIMA modeling. Clusters utilized in analysis are denoted by blue nodes

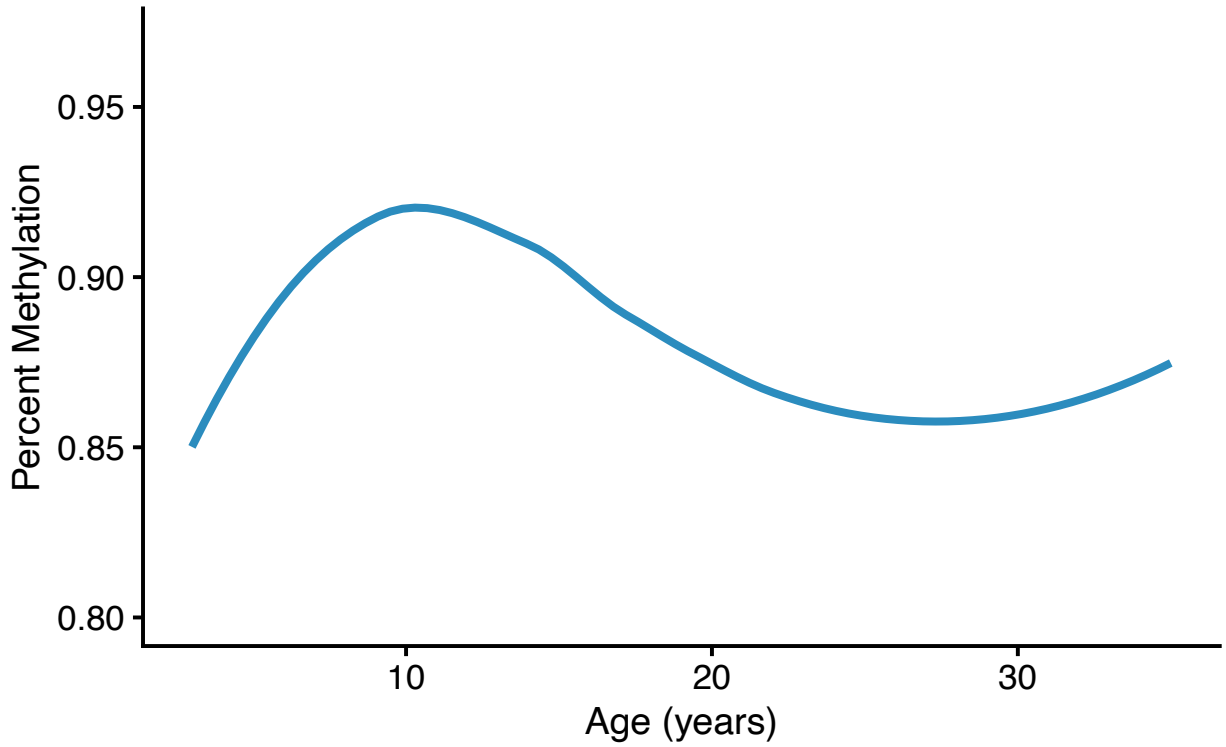


Fig. S3.3 Smoothed methylation trajectory for chr:77879817-77879847 as determined by ARIMA modeling



Fig. S3.4 Smoothed expression trajectory for *IGF1R* as determined from matched RNAseq samples

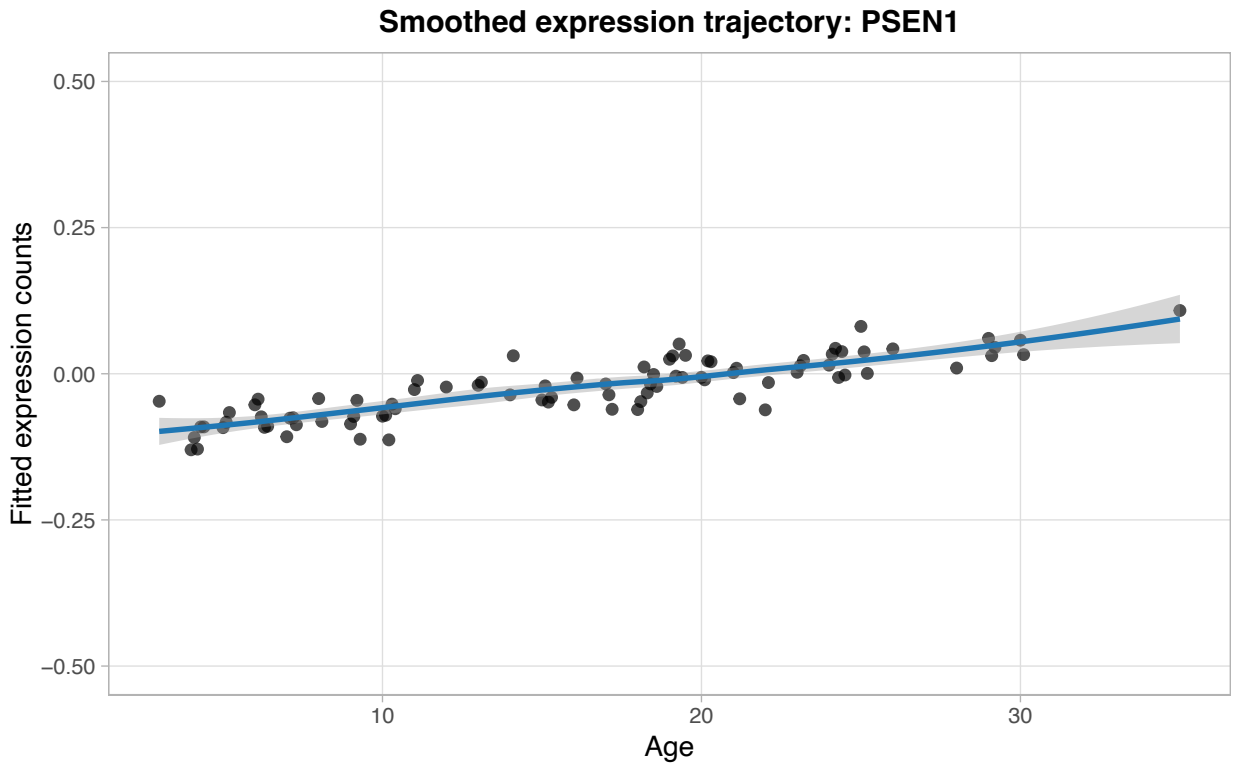


Fig. S3.5 Smoothed expression trajectory for *PSEN1* as determined from matched RNAseq samples

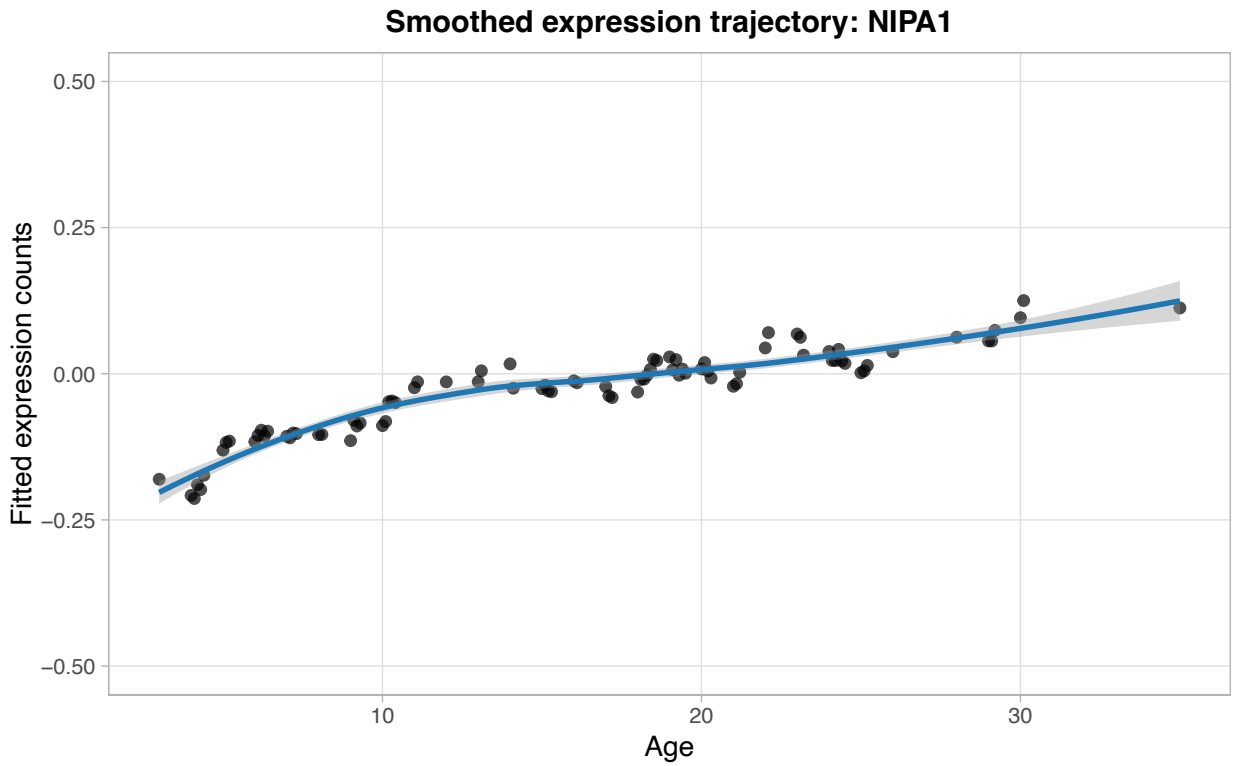


Fig. S3.6 Smoothed expression trajectory for *NIPA1* as determined from matched RNAseq samples

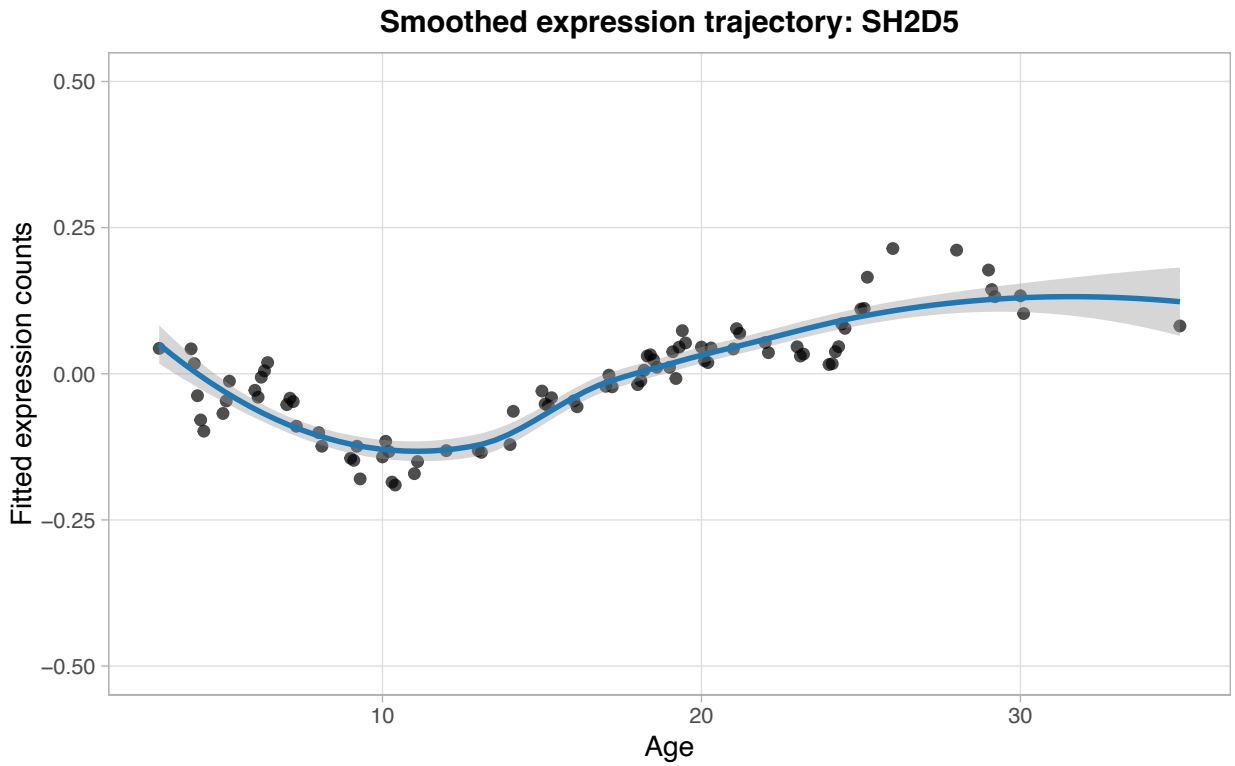


Fig. S3.7 Smoothed expression trajectory for *SH2D5* as determined from matched RNAseq samples

APPENDIX B

CHAPTER 2 SUPPLEMENTAL MATERIALS

Supplemental Methods

Preliminary data exploration

We performed preliminary data exploration using PCA analysis. The analysis highlighted a potential batch effect in the data along with the presence of outliers (**Fig. S2.1**).

To identify and address the potential technical effects in the data, we took the following steps. First, we tested whether there was presence of choroid plexus tissue contamination. The choroid plexus is a brain tissue responsible for making cerebrospinal fluid in the lateral and fourth ventricles of the brain (in close proximity to the hippocampus) and, due to its complex structure, it can be a source of contamination during routine dissections (Olney et al. 2022). Olney et al. (2022) identified the genes *TTR*, *FOLR1* and *PRLR* as being markers of choroid plexus contamination. An expression plot for these genes identified a subset (n=6) of individuals which exhibited higher than expected expression levels (**Fig. S2.2**).

Next, we performed hierarchical sample clustering analysis using a distance matrix to identify whether any individuals clustered distinctly from the rest of the dataset. The clustering analysis identified 2 individuals clustering distinctly from the remainder of the dataset (**Fig. S2.3**). Due to evidence of choroid plexus tissue contamination and high degree of outlier status, we chose to remove 8 individuals (4 female & 4 male) from the dataset and all subsequent analysis (**Table S2.1**).

Next, we performed variance partitioning analysis using the R package variancePartition (Hoffman and Schadt 2016). This provides another method of assessing the effects of metadata

variables on explaining the variance in the dataset. Our analysis identified the chip denotation – an identifier held over from sequencing – in our metadata as being the greatest explainer of the variance in the dataset compared to other metadata variables sex and age (**Fig. S2.4**). Therefore, we regressed out the effect of chip in our dataset and performed all subsequent analyses on the residuals.

To further assess potential residual technical confounding, we tested associations between the first 10 principal components (PCs) – representing the ~68% of cumulative variation in gene expression – and available technical variables, including RNA Integrity Number (RIN), Bioanalyzer Nano concentration, and RNA remaining after quality assessment. These analyses were performed using linear models on the subset of samples with available metadata (e.g., RIN data were available for 88 individuals, not all of whom were included in the differential expression analysis). After applying false discovery rate (FDR) correction for multiple testing, no significant associations were detected (FDR < 0.05), and these technical variables were not included as covariates in the differential expression models.

Differential expression analysis

To identify age-associated trajectories in hippocampal gene expression, we employed an ARIMA (Autoregressive Integrated Moving Average) modeling pipeline adapted from Márquez et al. (2020). ARIMA models are widely used in time series analysis for their ability to capture both autoregressive patterns and moving average effects, while accounting for non-stationarity through differencing. In the context of gene expression, this approach enables detection of dynamic, potentially nonlinear trends across chronological age (Brockwell and Davis 2002).

Normalized and residualized RNA-seq expression values (with effects of sex and technical

covariates regressed out) were first ordered by age. When multiple samples shared the same age, their expression values were averaged to ensure a single measurement per time point. These age-aligned gene expression series were then converted into regular time series objects using the zoo and ts frameworks in R. Each gene's time series was independently modeled using the auto.arima function from the forecast R package, which selects the best-fitting ARIMA configuration (p,d,q) based on the lowest Akaike Information Criterion (AIC) value. Optional Box-Cox transformations were applied to stabilize variance where appropriate. We retained models only if they met two quality control criteria: (1) inclusion of at least one autoregressive or moving average term (i.e., evidence of temporal structure), and (2) residuals that passed the Ljung-Box test for independence (FDR > 0.05), indicating good model fit without autocorrelated error. Genes that passed both filters were considered to exhibit non-random, age-associated trends in expression. To facilitate interpretation and comparison across ages, we applied LOESS (locally estimated scatterplot smoothing) interpolation to the fitted ARIMA values, generating smooth trajectories over a continuous age range. These smoothed predictions were used in downstream analyses of age-related expression patterns. This modeling approach is advantageous because it does not assume linearity or a fixed trend structure a priori, and it flexibly accommodates temporal complexity in gene regulation across the aging process.

Supplemental Results

Identification of DEGs in Female Dataset and Male Dataset

We aimed to assess whether the same molecular signals identified were held up when looking within sex. To address this, we divided our 88 individuals into males (N = 33) and females (N = 55) and reran our analyses. With these models, we identified 2,068 differentially expressed

genes in females and 1,742 genes in males (FDR < 0.05). Hierarchical clustering analysis suggested that the genes for both females (**Fig. S2.5A**) and males (**Fig. S2.5B**) grouped into 4 broad expression patterns.

Description of DEGs identified in the female dataset

Beginning with the 2,068 differentially expressed genes identified in the female dataset, the 4 broad expression patterns identified largely reflected what was reported in our previous ARIMA approach. Indeed ~80% of these genes overlapped with the genes identified in the previous approach. The first 2 clusters identified genes exhibiting linear trends in expression (**Fig. S2.6A and S2.6B**) while the other 2 identified genes exhibiting nonlinear trends in expression (**Fig. S2.6C and S2.6D**). Additionally, while the nonlinear clusters identified a shift in expression around ~10 years of age, they also identified shifts at ~19 and ~25 years (**Fig. S2.6C and S2.6D**).

Gene set and TF enrichment analyses for the genes in the linear clusters largely reflected the results reported in the original approach. For the nonlinear clusters, we were also able to identify significantly enriched GO terms after p-value correction. Specifically, for Female Cluster 3, the top 3 enriched GO terms were lipid modification (GO:0030258), mRNA splice site selection (GO:0006376) and positive regulation of establishment of protein localization to telomere (GO:1904851). TF enrichment contained additional enrichment for *DACH2*, a TF involved in cell fate determination and linked to premature ovarian failure (Bilgin and Kovanci 2015) (**Table S2.3**). For Female Cluster 4, the top 3 enriched GO terms were myelination (GO:0042552), positive regulation of transcription by RNA polymerase II (GO:0045944) and positive regulation of transcription, DNA-templated (GO:0045893) (**Table S2.6**). TF enrichment results largely mirrored those reported in the original ARIMA approach (**Table S2.3**).

Description of DEGs identified in the male dataset

For the 1,742 differentially expressed genes identified in the male dataset, the 4 broad expression patterns identified also reflected what was reported in the original ARIMA approach. Approximately ~65% of these genes overlapped with the genes identified in the original approach with the first 2 clusters identified exhibiting monotonic (i.e., linear) trends in expression while the other 2 exhibited nonlinear trends in expression. The nonlinear clusters also identified a shift in expression around ~10 years similar to our original approach (**Fig. S2.7**).

Gene set enrichment analysis provided slightly differing results compared to the previous approaches. For Male Cluster 1, the top 3 significantly enriched GO terms were identified as bleb assembly (GO:0032060), neutrophil degranulation (GO:0043312) and neutrophil activation involved in immune response (GO:0002283). For Male Cluster 2, the top 3 significantly enriched GO terms were regulation of cation channel activity (GO:2001257), regulation of neurotransmitter receptor activity (GO:0099601) and axonogenesis (GO:0007409). There were no significantly enriched GO terms identified for Male Cluster 3. Lastly, the top 3 significantly enriched GO terms for Male Cluster 4 were protein transport (GO:0015031), response to insulin (GO:0032868) and cellular response to insulin stimulus (GO:0032869) (**Table S2.7**). TF enrichment results for the genes in the male clusters largely upheld the patterns reported in the original and female specific ARIMA approach (**Table S2.3**).

Supplemental Discussion

Sex-specific trends in gene expression

The DEGs identified in the female- and male-specific ARIMA approaches largely uphold

the patterns identified in the original ARIMA approach. This is consistent with observed patterns of sex-biased gene expression in the macaque brain. Specifically, DeCasien et al. (2022) found that sex-biased differences in gene expression were more prevalent between brain regions rather than within regions. Therefore, we would not expect to see major differences between females and males in terms of the genes showing age-associated patterns of differential expression. Where we do observe differences is in how these genes are being used. Despite strong conservation, the timing and direction of expression differs between the sexes and highlights additional time points which may be relevant in shaping hippocampal aging within sex.

When examining the nonlinear clusters for both females and males, both identify a shift in expression centered around ~10 years of age, consistent with the combined dataset. The female nonlinear clusters also identified shifts in expression centered around the ages of ~19 and ~25 years (**Fig. S2.6**). Shifts in expression at ~19 and ~25 years of age could be explained by female individuals entering perimenopause and menopause respectively. Perimenopause and menopause have been demonstrated to significantly impact cognition and brain health in both humans and rhesus macaques (Greendale et al. 2011; Brinton et al. 2015; Sorwell et al. 2017; Ledford 2023). Specifically, perimenopause is characterized by disruptions in oestrogen production –a hormone important in glucose uptake and energy production (Ledford 2023). These disruptions contribute to a period of metabolic dysfunction as the brain must adjust to existence without oestrogen and indeed these perimenopausal changes are argued to be linked to Alzheimer's disease risk (Brinton et al. 2015; Wilson et al. 2011). Indeed, our TF enrichment results lend support to this explanation. Specifically, binding motifs for DACH2, a TF involved in cell fate determination and linked to premature ovarian failure (Bilgin and Kovanci 2015) were enriched among genes exhibiting nonlinear patterns of expression in the female-specific dataset.

While relevant hormone data and menses status for the specific rhesus macaque females included in this study were not available, an adjacent study examining changes in female reproductive hormones with age within rhesus macaque females housed at the ONPRC specifically found that, in general, female macaques showed normal to irregular or elongated cycles in their early to mid 20s while after 25 years they became intermittent (Downs and Urbanski 2006). As a result, the observed molecular shifts in older females could relate to changes in reproductive hormones/menses status associated with age.

An alternative explanation for the shift observed around ~25 years of age in females could have to do with the specific life history of rhesus macaques. The WNPRC classifies rhesus macaques over the age of 25 years as being post geriatric and "old age" which is reflected by a number of physiological changes including in the brain (Simmons 2016). We did not identify any additional shifts in expression in the male nonlinear clusters, however the lack of a signal in males does not preclude its existence. Our dataset is female biased and gets more female-biased with age (**Fig. 2.1A**). Indeed, there is only 1 male in our dataset >25 years of age. Therefore, we might observe a similar signal if older males were sampled.

Cell-type deconvolution results: impact and limitations

We did not observe a significant increase in microglia proportions associated across age as we might expect given increased activation and density of microglia with age in human brains (Edler et al. 2021). Additionally, microglia are a known cell-type demonstrated to be affected by the aging process at the gene expression level in the primate hippocampus (Zhang et al. 2021). However, this may not be entirely surprising given a handful of studies that indicate microglial activation and density differs in nonhuman primates compared to humans. Specifically, studies

concerning marmosets (Rodriguez-Callejas et al. 2016), rhesus macaques (Alan Peters et al. 2008; Kanaan et al. 2010; Chiou et al. 2022) and chimpanzees (Edler et al. 2017) show no significant change with age. Indeed, our results are consistent with these reports and appear to support the argument of a species-specific difference in microglia trajectory for humans. This argument is challenged however by conflicting reports which suggest older rhesus macaques (≥20 years) share a similar aging pattern to humans in terms of microglial densities (Robillard et al. 2016; Shobin et al. 2017). These inconsistencies highlight the need for further research in establishing normal age-related microglial changes amongst primates. Microglia serve as the resident immune cells of the brain and their increased activation contributes to increased inflammatory responses (Colonna and Butovsky 2017). With chronic inflammation being a hallmark of several neurodegenerative diseases (e.g., Alzheimer's disease) (Balin and Hudson 2014), establishing microglial trajectories in normal nonhuman primate brain aging may be necessary in investigating the human-specific vulnerability to these diseases.

An alternative explanation for the lack of change in overall microglia proportions could be that microglia activation associated with age may be driven by relative proportions of particular microglia subtypes (Paolicelli et al. 2022). Meaning, while there may not be significant changes in microglia proportions across age overall, certain subtypes relevant in brain aging/health might be. Of particular interest would be disease-associated microglia. Future studies should attempt to characterize microglia subtype changes with age to assess their role in shaping rhesus macaque hippocampal aging.

When performing our differential expression analysis while controlling for cell-type proportions, our results largely upheld the patterns identified in our original ARIMA approach (**Fig. S2.8** and **S2.9**). This suggests that age-related patterns in differential gene expression cannot

be completely explained by differences in cell-type proportions. Notably, the expression trajectories for all 4 identified clusters appear to exhibit less interindividual variation than the expression trajectories identified in the original approach (controlling for sex). This might suggest that fluctuations (i.e., seemingly dynamic changes between neighboring age points) may be explained by individual differences in cell type proportions rather than distinct cell-type changes with age. This in turn offers a more pointed view at age-related gene expression trajectories independent of interindividual variation in terms of cell-type proportions.

While revealing, cell-type deconvolution analyses have important limitations. Our approach infers cell-type, and our findings should be tested using more direct approaches (e.g., single-cell RNAseq). The estimated cell-type proportions and their changes over the lifespan are influenced by marker genes that themselves may be differentially expressed within cell types across the lifespan, thereby confounding our estimation of relative proportions. Additionally, to avoid introducing further error in cell-type predictions, we opted to focus here on predicting cell types at a broad level of cell class, precluding our ability to examine proportional changes in more granular, but potentially important cell types and subtypes. Therefore, we view the results, and their potential implications discussed here, as hypotheses for future research to investigate.

APPENDIX C

CHAPTER 3 SUPPLEMENTAL MATERIALS

Supplemental Results

CpG-level analyses reveal limited mean change and stabilization of methylation states associated with increased age

We identified 1,755 DMPs (~0.2% of total CpGs tested) significantly differentially methylated across age (FDR < 0.1), including 281 which gained methylation and 1,474 which lost methylation across age (**Table S3.2**); these DMPs were distributed across the genome (**Fig. 3.2A**). DMPs were found to be annotated primarily to intronic (12.5% in 1st introns; 29.9% in other introns) and distal intergenic regions (26.4%) and enriched for processes related to nervous system development (GO:0007399), anatomical structure morphogenesis (GO:0009653) and regulation of transcription by RNA polymerase II (GO:0006357). No significantly enriched KEGG pathways were identified. Additionally, motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs. Across significant DMPs, deviation from 0.5 methylation decreased across age ($\beta = -0.00173$, 95% CI [−0.00194, −0.00148], $p = 2.2e{-16}$) (**Fig. 3.2B**), with a significant mean correlation of −0.18 across sites (Wilcoxon signed-rank test, $p = 6.1e{-81}$) (**Fig. 3.2C**). This is consistent with known trends of methylation at age-associated DMPs shifting towards an intermediate state (Seale et al. 2022).

When looking specifically at DMPs which gained and lost methylation, DMPs which gained methylation were most common in promoter (≤3 kb; 43.1%) and distal intergenic regions (32.4%) while DMPs which lost methylation were most common in intronic (13.5% in 1st introns; 33.1% in other introns) and distal intergenic regions (25.3%). DMPs which gained methylation

were found to be enriched for processes related to animal organ morphogenesis (GO:0009887), animal organ development (GO:0048513) and regulation of transcription by RNA polymerase II (GO:0006357); DMPs which lost methylation were enriched for processes related to nervous system development (GO:0007399), regulation of signal transduction (GO:0009966) and anatomical structure morphogenesis (GO:0009653) (**Table S3.3**). No KEGG pathways were significantly enriched for either subset of DMPs. Motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs for either subset DMPs.

Given prior evidence of widespread sex-specific patterns in DNA methylation aging (Masser et al. 2017; Chinn et al. 2021), we next examined whether the molecular signatures of age-related methylation change differed between females (n = 56) and males (n = 30) by repeating our analyses within sex. We identified 511 DMPs (89 gained methylation and 422 lost methylation) in females and 327 DMPs (75 gained methylation; 252 lost methylation) in males; only 15 of these DMPs were found to be conserved between sexes (**Table S3.3**). Female DMPs were primarily annotated to intronic (11.4% in 1st introns; 29.2% in other introns) and promoter regions (≤3 kb; 27.6%) and enriched for processes related to nervous system development (GO:0007399), regulation of cellular component organization (GO:0051128) and microtubule-based process (GO:0007017). No significantly enriched KEGG pathways were identified for female DMPs. Male DMPs were primarily annotated to intronic (12.5% in 1st introns; 31.8% in other introns) and distal intergenic regions (30.0%) and enriched for processes related to cytoskeleton organization (GO:0007010) and intracellular signal transduction (GO:0035556); enriched KEGG pathways for male DMPs included focal adhesion (KEGG:04510), thyroid hormone signaling pathway (KEGG:04919) and Fc gamma R-mediated phagocytosis (KEGG:04666). Motif enrichment analysis identified only 1 significantly enriched motif for female DMPs, Bach2, a transcriptional

repressor suggested to be implicated in the aging-mediated functional decline of dentate gyrus neurons and regulator of synaptic plasticity genes in the aging mouse hippocampus (Bilgic et al. 2025). Motif enrichment analysis did not identify any significant overrepresentation of binding motifs for TFs in male DMPs.

CpG-level analyses reveal widespread variance remodeling across age

Our variance analysis identified 237,531 VMPs (29.6% of total CpGs tested; FDR < 0.1) across age. Of these, 227,068 VMPs (95.6%) increased in variance (mean LogVarRatio = 0.18; median = 0.20) while 10,463 (4.4%) decreased in variance across age (mean LogVarRatio = -0.03, median = -0.026) (**Fig. 3.3** and **Table S3.4&5**). These results are consistent with known trends of increased variance in DNAm associated with aging (Fernández et al. 2015; Hannum et al. 2013; Oh et al. 2016; Phipson and Oshlack 2014; Vershinina et al. 2021; Jeong et al. 2025). VMPs were primarily annotated to promoter (≤3 kb; 34.4%) and intronic regions (8.3% in 1st introns; 23.8% in other introns) and enriched for processes related to positive regulation of macromolecule metabolic process (GO:0010604), intracellular signal transduction (GO:0035556) and nervous system development (GO:0007399); enriched KEGG pathways included glutamatergic synapse (KEGG:04724), MAPK signaling pathway (KEGG:04010) and axon guidance (KEGG:04360). Motif enrichment analysis identified significant overrepresentation of binding motifs for 142 TFs. The top enriched motif was for Ronin, a TF regulating mitochondrial function genes demonstrated to be important in CNS development in mice (Poché et al. 2016). Other enriched motifs included binding sites for homeobox TFs (e.g., CRX; Nkx3.1; Oct11) zinc fingers (e.g., Maz; Snail1; ZEB1; ZEB2) TFs implicated in synaptic plasticity (e.g., Atf1; CRE; CREB5).

VMPs that increased in variance across age were primarily annotated to promoter (≤3 kb; 35.1%) and intronic regions (8.2% in 1st introns; 23.7% in other introns) and enriched for biological processes including positive regulation of macromolecule metabolic process (GO:0010604), intracellular signal transduction (GO:0035556) and nervous system development (GO:0007399); enriched KEGG pathways included MAPK signaling pathway (KEGG:04010), glutamatergic synapse (KEGG:04724) and axon guidance (KEGG:04360). Motif enrichment analysis identified significant overrepresentation of binding motifs for 129 TFs. The top enriched motif again was for Ronin. Other enriched motifs included binding sites for zinc fingers (e.g., KLF3; Snail1; ZEB1; ZEB2), TFs implicated in neural progenitor differentiation (e.g., E-box; E2A; TFE3) and synaptic plasticity (e.g., Atf1; CRE; CREB5) (**Table S3.5**).

VMPs which decreased in variance across age were primarily annotated to distal intergenic (39.0%) and intronic regions (10.5% in 1st introns; 25.5% in other introns) and were enriched for processes related to anatomical structure morphogenesis (GO:0009653), nervous system development (GO:0007399) and neurogenesis (GO:0022008); enriched KEGG pathways included adrenergic signaling in cardiomyocytes (KEGG:04261), dilated cardiomyopathy (KEGG:05414) and calcium signaling pathway (KEGG:04020). Motif enrichment analysis identified significant overrepresentation of binding motifs for 110 TFs. The top enriched motif was for Etv2, a TF involved in the vascularization in the developing brain (Cakir et al. 2019). Other enriched motifs included binding sites for other ETS factors (e.g., EHF; ELF3; ERG; ETS1; ETV1; PU.1), Homeobox TFs (e.g., Hoxc6; Meis1; Nanog; Nkx2.2; Nkx2.5), bHLH family TFs (e.g., Atoh1; NeuroD1; Olig2; Twist2) and zinc finger proteins (e.g., KLF1; KLF17; Maz; Sp1; Sp2; ZNF341) (**Table S3.5**). Several of these TFs (e.g., FOXO1 & Nkx2.2) have also been linked to neurodegenerative disease pathways, including Parkinson's and AD (Mio et al. 2023; Du and

Zheng 2021).

Additionally, when looking within sex, we found 181,489 VMPs (173,264 increased in variance; 8,225 decreased in variance) in females and 138,171 VMPs (131,063 increased in variance; 7,108 decreased in variance) in males; 60,846 VMPs were found to overlap between sexes (**Table S3.6**). Female VMPs were primarily annotated to promoter (≤3 kb; 37.0%) and intronic regions (7.9% in 1st introns; 22.5% in other introns) and enriched for processes related to positive regulation of macromolecule metabolic process (GO:0010604), anatomical structure morphogenesis (GO:0009653) and intracellular signal transduction (GO:0035556); enriched KEGG pathways included MAPK signaling pathway (KEGG:04010), glutamatergic synapse (KEGG:04724) and endocytosis (KEGG:04144). Male VMPs were primarily annotated to promoter (≤3 kb; 38.7%) and intronic regions (7.8% in 1st introns; 21.5% in other introns) and enriched for processes related to positive regulation of macromolecule metabolic process (GO:0010604), intracellular signal transduction (GO:0035556) and nervous system development (GO:0007399); enriched KEGG pathways included endocytosis (KEGG:04144), axon guidance (KEGG:04360) and endocrine resistance (KEGG:01522). Motif enrichment analysis identified 150 significantly enriched motifs for female VMPs and 186 motifs for male VMPs. The top enriched motif for female VMPs was Ronin. Other enriched motifs included promoter motifs regulating transcriptional initiation (e.g., GFY; NFY; TATA-Box), zinc finger proteins (e.g., KLF3; KLF4; Snail1; ZNF148) and TFs implicated in synaptic plasticity (e.g., Atf1; CRE; CREB5). The top enriched motif for male VMPs was NFY, a TF which recognizes the CCAAT box in gene promoters to regulate transcription (Kahle et al. 2005). Other enriched motifs included zinc finger proteins (e.g., KLF3; KLF4; Snail1; Maz), TFs implicated in synaptic plasticity (e.g., Atf1; CRE; CREB5) and circadian regulators (e.g., CLOCK).

Sex-specific region-level analyses

Female age-associated regions were primarily annotated to intronic (11.0% in 1st introns; 25.3% in other introns) and distal intergenic regions (30.8%) and were enriched for processes related to nervous system development (GO:0007399), generation of neurons (GO:0048699) and anatomical structure morphogenesis (GO:0009653); enriched KEGG pathways included synaptic vesicle cycle (KEGG:04721), efferocytosis (KEGG:04148) and cholinergic synapse (KEGG:04725). Male age-associated regions were primarily annotated to distal intergenic (34.8%) and intronic regions (11.1% in 1st introns; 23.3% in other introns) and were enriched for processes related to anatomical structure morphogenesis (GO:0009653), intracellular signal transduction (GO:0035556) and cell adhesion (GO:0007155); enriched KEGG pathways included focal adhesion (KEGG:04510), synaptic vesicle cycle (KEGG:04721) and efferocytosis (KEGG:04148).

Supplemental Discussion

Our findings align with prior research on DNAm changes during brain aging. The observed loss of methylation across the genome (**Fig. 3.2A and Fig. 3.4B**), evident in both DMPs and age-associated regions, is consistent with reports from rodent and human hippocampus showing widespread methylation loss with age (Unnikrishnan et al. 2018; Yazar et al. 2023). However, while such loss of methylation is a reproducible hallmark of aging, it is not the predominant driver of epigenetic remodeling. Instead, the aging methylome is defined primarily by increased variance and stochastic drift (i.e., localized, cell- and region-specific methylation changes that accumulate over time and create inter-individual heterogeneity) as evidenced by our VMPs (Unnikrishnan et al. 2018). These stochastic alterations are interspersed with targeted hypermethylation events, particularly within regulatory regions of genes involved in synaptic plasticity, immune regulation

and brain disease, suggesting that aging reflects both loss of global methylation maintenance and regulatory control of key gene networks (Yazar et al. 2023; Chinn et al. 2021).

The increased variance associated with increased age, as evidenced by our VMPs, is also consistent with known trends. Multiple studies in humans and model organisms have shown that aging is accompanied by a global increase in methylation variability, reflecting a progressive loss of epigenetic maintenance fidelity and the accumulation of stochastic methylation changes, a phenomenon termed epigenetic drift (Teschendorff et al. 2013; Fernández et al. 2015; Hannum et al. 2013; Oh et al. 2016; Phipson and Oshlack 2014; Vershinina et al. 2021; Jeong et al. 2025). Such drift is thought to arise from replication errors, reduced DNA methyltransferase efficiency, and cumulative environmental exposures, resulting in growing heterogeneity across individuals and cell populations (Fraga 2009; López-Gil et al. 2023; Seale et al. 2022; Teschendorff et al. 2013). Compared to DMPs, the greater proportion of VMPs we observe suggests that stochastic mechanisms may play an important role in shaping the epigenomic landscape during hippocampal aging. Similar patterns of increased methylation variance with age have been reported in the human brain (Oh et al. 2016; Jeong et al. 2025) and other tissues (Hannum et al. 2013; Phipson and Oshlack 2014), often coinciding with reduced transcriptional precision. In our study, VMPs showing increased variance across age were predominantly located in promoter and distal intergenic regions and were enriched for pathways related to metabolic processes (GO:0010604), transcriptional regulation (GO:0006366), and nervous system development (GO:0007399) (**Table S3.5**). Together, these results indicate that increased methylation variance across age primarily affects loci involved in transcriptional control and synaptic signaling, reflecting reduced epigenetic stability in processes important for hippocampal function during aging.

Concurrently, we found DMPs which gained methylation across age to be predominantly annotated to promoter regions and enriched for processes broadly related to cellular plasticity in the hippocampus (e.g., central nervous system neuron differentiation [GO:0021953]; cell fate specification [GO:0001708]) (**Table S3.3**), consistent with known trends of CpG methylation gains across age (Teschendorff et al. 2010; Day et al. 2013; Rakyan et al. 2010; Voisin et al. 2021; Chiavellini et al. 2022). Additionally, DMPs which lost methylation across age were predominantly annotated to intronic and distal intergenic regions and enriched for processes related to synaptic plasticity (e.g., nervous system development [GO:0007399]; neuron differentiation [GO:0030182]; regulation of synaptic plasticity [GO:0048167] (**Table S3.3**), also consistent with known trends of CpG methylation loss in those regions across age (Fernández et al. 2015; Pérez et al. 2018; Chinn et al. 2021). These findings support a model in which promoter methylation gains contribute to gradual restriction of cellular plasticity with age, while methylation loss of intronic and enhancer-like regions sustains activity-dependent transcriptional programs that promote synaptic resilience during hippocampal aging although this exact relationship would need to be functionally validated.

Methylation values for DMPs also drift towards an intermediate (50%) across age (**Fig. 3.2B and 3.2C**). This is consistent with established patterns that CpG sites which are highly methylated or unmethylated in youth become more predictable and less ordered at older ages (Hannum et al. 2013; Field et al. 2018). Therefore, aging is argued to be associated with a reduction in fidelity of epigenetic maintenance systems (M. J. Jones et al. 2015; Hayflick 2007). This phenomenon can also be observed in the methylation trajectories identified by the ARIMA approach. Specifically, the majority of age-associated regions exhibit patterns of methylation loss across age entirely (**Fig. 3.4B**) and in a nonlinear fashion after ~10 years of age (**Fig. 3.4C and**

3.4D). These methylation trajectories also converge towards an intermediate level (50%) across age, underscoring a progressive erosion of epigenetic stability that characterizes the aging process (Seale et al. 2022).

Many studies have reported an age-related decline in *DNMT1* expression in the hippocampus of rodents (Elsner et al. 2013; P. Singh and Thakur 2014), consistent with the role of DNA methyltransferases in regulating synaptic plasticity and memory formation (P. Singh and Paramanik 2025). In humans, reduced *DNMT1* expression has also been linked to neurodegenerative disease; for example, *DNMT1* levels are decreased in neurons of the entorhinal cortex layer II in AD patients compared to age-matched controls (Mastroeni et al. 2010). In contrast, we observed an opposite pattern in matched samples, with increasing *DNMT1* expression across age in the hippocampus (T. J. Anderson et al. 2025). This finding aligns with broader evidence that macaques maintain higher expression of genes involved in neurogenesis and synaptic plasticity into adulthood (T. Zhu et al. 2018). Sustained or elevated *DNMT1* expression in the macaque brain may therefore contribute to preserved plasticity and resilience, and the divergence between species underscores the importance of defining molecular signatures of aging in nonhuman primates to better understand human-specific vulnerability to neurodegenerative disease.

Cluster 2, while exhibiting predominantly monotonic loss of methylation across the adult lifespan, showed a modest late-life deviation from this trend in the LOESS-smoothed trajectory, occurring near the upper range of our sampled ages (~29-30 years) (**Fig. 3.4B**). Because our dataset is cross-sectional, we cannot determine whether this shift represents true within-individual re-methylation or distinct epigenetic profiles of extremely aged or surviving individuals. Additionally, our dataset contains a limited number of individuals represented at advanced ages

(**Fig. 3.1**) meaning this pattern may reflect reduced sampling density rather than a distinct biological change. Nonetheless, late-life attenuation or partial reversal of age-associated methylation loss has been reported in other systems. For example, centenarians exhibit CpG sites that display relative methylation gains compared to elderly controls, particularly within H3K9me3-marked heterochromatin and developmental gene promoters (Xiao et al. 2023; Heyn et al. 2012), and late-life methylation gains have been described in aged rodent hippocampus and human frontal cortex, particularly in very old individuals (>80 years in humans) (Chiavellini et al. 2022; D. Sun and Yi 2015). In humans, multi-omic profiling of hippocampal tissue from older adults with exceptional episodic memory ("SuperAgers") compared with age-matched controls and individuals with AD pathology revealed sustained signs of hippocampal neurogenesis accompanied by distinct chromatin accessibility signatures in neuron populations (Disouky et al. 2026). These findings suggest that late-life maintenance of neurogenic potential and chromatin regulatory states may be linked to preserved cognitive resilience, highlighting the broader regulatory role that epigenomic elements, including DNA methylation and chromatin accessibility, play in shaping the trajectory of brain aging in humans. However, the genomic distribution and mechanistic basis of these changes remain incompletely characterized, and direct comparisons across species are limited by differences in sampling structure and lifespan representation. Taken together, the late-life deviation observed in our data may reflect technical limitations, survivorship effects, or biologically meaningful stabilization of epigenetic states in advanced age. Disentangling these possibilities will require denser sampling at the oldest ages and, ideally, longitudinal epigenetic profiling in long-lived primates.

Rhesus macaques over the age of 25 years are often classified as post geriatric and "old age" which is reflected by a number of physiological changes including in the brain (Simmons

2016). If the late-life deviation observed in Cluster 2 reflects a biological phenomenon rather than sampling artifact, it may coincide with regulatory adjustments occurring during advanced age. Regions within this cluster were enriched for pathways broadly related to neural development and cellular maintenance, including axon guidance (KEGG:04360) and neuron differentiation (GO:0030182) (**Table S3.7**) which are processes directly implicated in modulating synaptic plasticity. Rather than representing residual developmental programs, these enrichments may indicate sustained or re-engaged regulatory networks that remain functionally relevant in the aging primate hippocampus. Motif enrichment within Cluster 2 further identified NeuroG2, a transcription factor involved in neuronal differentiation and survival (Deng et al. 2010), suggesting that regions exhibiting progressive loss of methylation across much of the adult lifespan may remain associated with regulatory programs linked to neuronal maintenance. While these findings do not demonstrate adaptive stabilization, they raise the possibility that sustained methylation loss at specific regulatory loci could influence pathways relevant to late-life neural function. Clarifying whether such patterns reflect compensatory regulation, selective survival or simply stochastic effects will require targeted investigation in more densely sampled aged cohorts.

While functional enrichment across nonlinear clusters was modest, we observed enrichment for processes related to intracellular trafficking and vesicle transport (**Table S3.7**). These pathways are central to synaptic function and cellular signaling, suggesting that nonlinear methylation shifts may intersect with age-related changes in intercellular communication, a recognized hallmark of aging (López-Otín et al. 2023). The only significantly enriched KEGG pathway among nonlinear regions was type II diabetes mellitus (KEGG:04930), raising the possibility that insulin-resistance-related mechanisms may intersect with nonlinear DNAm trajectories. Indeed, molecular alterations in insulin resistance pathways within the hippocampus

have been shown to impact synaptic plasticity (Spinelli et al. 2019), although evidence for a nonlinear relationship between these processes and aging is limited. However, given the limited breadth of enrichment and absence of strong motif signals, the biological underpinnings of these inflection points remain unclear, and regions exhibiting nonlinear trajectories may represent functionally heterogeneous or incompletely annotated regulatory loci.

Candidate regulatory regions highlighted presenilin I (*PSEN1*) as a potential methylation-sensitive locus. Age-associated region chr7:135272269-135272542 exhibits a progressive loss of methylation across age and overlaps the first intron of *PSEN1*, which we also previously found to be upregulated across age in the same individuals (T. J. Anderson et al. 2025) (**Fig. S3.5**). *PSEN1* mutations are causally linked to familial early-onset AD (Cochran et al. 2023; Tang and Ruan 2025; Yan et al. 2024; Arber et al. 2021; Petit et al. 2022; Orozco-Barajas et al. 2022), but *PSEN1* also plays essential roles in hippocampal neurogenesis and synaptic plasticity (S. H. Lee et al. 2023; Bonds et al. 2015). In mice, reduced *PSEN1* expression impairs neurogenesis and learning (Bonds et al. 2015). The convergence of methylation loss and increased expression of *PSEN1* suggests that age-associated epigenetic remodeling at this locus may be functionally relevant in the aging hippocampus.

When examining candidate regulatory regions derived from clusters exhibiting nonlinear methylation trajectories, we identified loci that overlap promoters of genes with temporally dynamic expression patterns. For example, age-associated region chr7:639035-639527 exhibited a nonlinear methylation trajectory where methylation levels typically increase before reaching a peak centered around ~10 years of age before decreasing in methylation and overlapped the promoter of NIPA magnesium transporter I (*NIPA1*), a gene encoding a magnesium transporter involved in nervous system development and maintenance (Goytain et al. 2007) whose expression we found to

increase across age in these individuals (T. J. Anderson et al. 2025) (**Fig. S3.6**). Pathogenic dominant variants in *NIPA1* cause SPG6 hereditary spastic paraplegia, a neurodegenerative disorder characterized by progressive motor impairment (Rainier et al. 2003; Goytain et al. 2007), underscoring the functional relevance of this locus in neural integrity.

Similarly, age-associated region chr1:203983149-203983234 displayed a comparable nonlinear methylation trajectory and midlife inflection and overlapped the promoter of SH2 domain containing 5 (*SH2D5*), which encodes a brain-enriched adaptor-like protein implicated in synaptic function and neurological disorders such as epilepsy (H. Guo et al. 2025). Notably, *SH2D5* exhibited a nonlinear expression trajectory with a midlife inflection in the same individuals (T. J. Anderson et al. 2025) (**Fig. S3.7**), mirroring the shift observed in DNAm. The temporal concordance between promoter methylation dynamics and gene expression trajectories at these loci suggests that midlife epigenetic remodeling may intersect regulatory programs that are particularly sensitive to age-associated transitions in the primate hippocampus. However, these relationships remain correlative, and functional validation will be necessary to determine whether age-associated methylation changes directly modulate transcription at these loci.

Although genic intersections highlighted candidate *cis*-regulatory loci linked to age-associated transcriptional change, a substantial fraction of age-associated DNAm regions were annotated to distal intergenic features (30.3%) (**Table S3.7**), motivating a focused evaluation of whether these age-associated intergenic regions coincide with distal regulatory elements. Consistent with this idea, many intergenic regions overlapped annotated human enhancer elements (**Fig. 3.5B** and **Table S3.10**), identifying intergenic loci where age-associated methylation remodeling intersects distal *cis*-regulatory elements with the capacity to influence transcription.

Across genomes, many TEs are kept transcriptionally silent by repressive chromatin and

epigenetic mechanisms, including DNA methylation. Previous research suggests that this silencing can erode with age, contributing to increased TE permissiveness in some contexts (Peze-Heidsieck et al. 2022; Merenciano et al. 2025). In our dataset, many intergenic regions overlapping TEs exhibit age-associated loss of methylation across the adult lifespan (**Fig. 3.5D**). This pattern is consistent with a model in which age-related loss of epigenetic constraint could increase regulatory "noise" at TE-rich loci and potentially enable TE transcription or TE-linked regulatory activity in the aging and/or diseased brain (Y. Feng et al. 2024; Le Breton et al. 2024). Notably, among TE-overlapping age-associated intergenic regions, *Alu* subfamilies were particularly abundant (e.g., *Alu*Sc8; *Alu*Sg; *Alu*Sx1; *Alu*Y) (**Fig. 3.5E**). *Alu* transcripts have been demonstrated to increase in expression in brain tissues with age in the extracellular vesicles of humans and their expression is highly associated with plasma neurofilament light chain suggesting a link between *Alu* expression in neuronal injury (S. Yu et al. 2025). Additionally, researchers have observed increases in putative somatic *Alu*Y insertions, our most abundantly overlapped TE, in individuals with late-stage AD compared to controls suggesting that these elements, especially younger *Alu* subfamilies, are associated with advanced neurodegeneration in humans (Ramirez et al. 2025). Together, these observations motivate follow-up work to test whether age-associated methylation changes at TE-overlapping intergenic loci is accompanied by measurable changes in TE expression, chromatin accessibility, or local transcriptional effects in the primate hippocampus.

APPENDIX D

CHAPTER 4 SUPPLEMENTAL MATERIALS

Supplemental Results

Sex-specific miRNA modeling

Overlap analysis revealed limited shared signal across the two sex-specific sets and modest overlap to the original approach. Of the 24 female age-associated miRNAs, 15 (62.5%) were also identified in the original analysis, while 40 of the 91 male age-associated miRNAs (44.0%) overlapped with the original analysis. Only 3 miRNAs were shared between the male and female age-associated sets (2.7% overlap), with 49 miRNAs unique to males and 7 unique to females relative to both the other sex and the original analysis.

To assess the functional implications of these sex-specific miRNA sets, we identified high-confidence predicted gene targets (miRDB score ≥ 90) for each analysis separately. The original set of 91 age-associated miRNAs yielded 5,012 miRNA-gene pairs spanning 3,429 unique predicted target genes, while the male-specific set of 91 miRNAs yielded 3,107 pairs spanning 2,533 unique targets and the female-specific set of 24 miRNAs yielded 949 pairs spanning 889 unique targets. Overlap analysis at the gene target level revealed that 1,918 targets (46.8% of the combined union) were shared between the male-specific and original analysis, while 786 targets (19.2%) were shared between the female-specific and original analysis, and 349 targets (8.5%) were shared between the two sex-specific sets. Only 303 genes (7.4%) were predicted targets in all three analyses. Notably, 569 genes were unique to the male-specific analysis and 57 were unique to the female-specific analysis relative to both the other sex and the original set, while 46 genes were predicted targets of sex-specific miRNAs in both males and females but were not identified in the

original combined analysis.

APPENDIX E

CHAPTER 5 SUPPLEMENTAL MATERIALS

Supplemental Methods

Briefly, regions were defined using the regionFinder3 function in bsseq (Hansen et al. 2012), merging CpGs, from an initial matrix of 802,283 CpGs, located within 250 bp. This proximity threshold was determined to be optimal for clock construction when compared to other region construction approaches. For each region, percent methylation was calculated as the ratio of methylated to total reads. Regions were retained if they showed moderate methylation (average between 10–90%) and complete coverage across samples resulting in a matrix of 57,599 regions that was used as input. The caret package (Kuhn 2008) was used to optimize the alpha parameter for elastic net regularization. Using the optimized alpha value, the final clock model was trained with a Leave-One-Out Cross-Validation (LOOCV) approach. In each iteration of LOOCV, one sample was held out as the test set while the remaining samples were used to train the model. A penalized regression model was fit to predict chronological age from methylation data, and the trained model was then used to predict the age of the held-out sample. This process was repeated iteratively until every sample had been left out once, ensuring robust performance evaluation. Beta coefficients were extracted from each iteration to identify regions contributing to age prediction. The final model was evaluated by comparing predicted versus actual ages using linear regression and correlation analysis.

Interestingly, we found that altering the regionFinder3 size parameter greatly impacted clock performance. When examining clock models constructed with different filtering parameters, we found an optimal region size to be 250bp for our hippocampus-specific clock (**Fig. 5.1**)

compared to clocks constructed using region sizes of 100bp (Pearson's r = 0.86, 95% CI: 0.79–0.91, p = 2.2e-16; MAE = 3.10 years), 500bp (Pearson's r = 0.90, 95% CI: 0.84–0.93, p = 2.2e-16; MAE = 2.94 years) and 1000bp (Pearson's r = 0.86, 95% CI: 0.79–0.91, p = 2.2e-16; MAE = 3.11 years). The improved performance at a 250 bp region size likely reflects the local structure of CpG co-methylation across the genome. CpG sites often exhibit correlated methylation changes within short genomic domains, typically spanning a few hundred base pairs (Ochana et al. 2025). Regions smaller than this may fail to capture these coordinated patterns, while larger regions may combine distinct regulatory contexts and introduce noise.

Supplemental Results

In parallel, we identified 6,584 differentially methylated CpGs (FDR < 0.1) between rapamycin-treated and control individuals, including 4,610 sites which showed methylation gains and 1,974 sites which showed methylation losses in treated individuals. CpGs were predominantly located in intronic and distal intergenic regions, with the majority positioned 10–100 kb from a transcription start site (TSS). Ontology enrichment analysis for CpGs which gained methylation identified regulation of developmental process (GO:0050793), positive regulation of nucleobase-containing compound metabolic process (GO:0045935), and positive regulation of RNA metabolic process (GO:0051254) as the top enriched terms, with KEGG pathways including Arginine and proline metabolism (KEGG:00330), Oxytocin signaling pathway (KEGG:04921) and Axon guidance (KEGG:04360). For CpGs which lost methylation, enriched GO terms included nervous system development (GO:0007399), cell-cell signaling (GO:0007267), and cytoskeleton organization (GO:0007010), with KEGG pathways such as Calcium signaling (KEGG:04020), Dopaminergic synapse (KEGG:04728), and Circadian entrainment (KEGG:04713). Together,

these findings suggest that rapamycin treatment is associated with widespread remodeling of regulatory regions and pathways relevant to brain function, even in the absence of a statistically significant shift in epigenetic age.

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