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Characterization of YY1, RUNX1, NFIB, and PPARG:
A Study in Aged Human Induced Neurons

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Yatavee Vajaphattana

Committee in charge:

Professor Fred H. Gage, Chair
Professor Nicholas C. Spitzer, Co-Chair
Professor Gulcin Pekkurnaz

2023

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University of California San Diego

2023

DEDICATION

This work is dedicated to my family.

You are the reason behind my success.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
TF	Transcription Factor
iNs	Induced Neurons
DEGs	Differentially Expressed Genes
DEs	Differentially Expressed
PCA	Principal Component Analysis
Log ₂ FC	Log ₂ (Fold Change)
p _{adj}	Adjusted p-value

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All of the work included in this thesis is under consideration for a publication currently being prepared for submission. Yatavee is a researcher working under Dr. Raffaella Lucciola and Dr. Fred H. Gage, and co-author of this material. Yatavee contributed to this publication by performing experiments and data collection, as well as data analysis for the morphology experiment.

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ABSTRACT OF THE THESIS

Characterization of YY1, RUNX1, NFIB, and PPARG:
A Study in Aged Human Induced Neurons

by

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Master of Science in Biology

University of California San Diego, 2023

Professor Fred H. Gage, Chair
Professor Nicholas C. Spitzer, Co-Chair

Alzheimer's Disease (AD) is a progressive neurodegenerative disease regarded as a multifactorial disorder, a condition caused by multiple factors. With the majority of AD patients aged 65 and older, age is one of the strongest risk factors for AD, but its causes are not fully understood. Previous transcriptomic profiling of AD neurons identified a list of differentially expressed genes unique to AD; YY1, RUNX1, NFIB, and PPARG emerged as some of the most upregulated transcription factors (TFs) reportedly associated with biological dysfunctions phenotypic of AD in various cell types. However, their function in aged neurons remains unknown. To investigate the

possible contributions of YY1, RUNX1, NFIB, and PPAR γ to AD pathology, we questioned their ability to reproduce AD-related phenotypes in aged neurons by characterizing transcriptomic changes caused by individual overexpression of each TF. We utilized induced neurons (iNs) produced from the reprogramming of old, healthy, donor-derived fibroblasts directly into neurons as a human-relevant means of capturing the age. All four TFs retain aspects of their previously described functions in old iNs with YY1 regulating the metabolic program, RUNX1 restricting neuronal identity, PPAR γ regulating lipid metabolism and inflammation, and NFIB promoting the expression of developmental genes, possibly facilitating oncogenic transformations and contributing to the vulnerable hypo-mature state seen in AD neurons. Further investigation confirmed that YY1 and RUNX1's transcriptomic effects are functionally translatable, identifying them as potential targets for medical intervention.

INTRODUCTION

Alzheimer's Disease and the Downfall of The Amyloid Cascade Hypothesis

Alzheimer's Disease (AD) is an irreversible, progressive neurodegenerative disease characterized by neuronal cell death, cognitive dysfunction, and memory decline among other symptoms (Bondi et al., 2017). Considered the most common form of dementia and the sixth leading cause of death in 2019 according to the CDC, it is estimated that 6.7 million Americans over the age of 65 are living with AD in 2023 ("Alzheimer's Disease Facts and Figures," 2023)

First characterized by Dr. Alois Alzheimer in 1907, brain atrophy, amyloid plaques, and neurofibrillary tangles (NFTs) are the pathological hallmarks of AD but what exactly causes the development of AD is still not understood (Alzheimer, 1907). NFTs, found within the soma of the neuron, are formed from the aggregation of hyperphosphorylated tau (Bondi et al., 2017). In a healthy person, the axonal protein tau stabilizes microtubules that help neurons retain their structure and aid in intracellular transport (Binder et al., 1985; Lindwall & Cole, 1984). In the AD brain, tau is hyperphosphorylated and loses its affinity for the microtubules. Tau detaches from the microtubules and aggregates to form tangles (Lee et al., 1991; Geodert & Jakes, 1990). Without the stabilization of tau, the microtubules disintegrate, causing the loss of intraneural connections and ultimately, neuronal death (Bierer et al., 1995). Once formed, the NFT can spread from neuron to neuron through the synapse and cause similar atrophy in other areas of the brain (Dujardin et al., 2014).

Similarly, amyloid plaques are formed from the aggregation of misfolded amyloid beta ($A\beta$) proteins. The amyloid precursor protein (APP) is normally cleaved by α -secretase to produce soluble $A\beta$. However, in the diseased brain, APP is cleaved at alternate sites by β and γ -secretase, resulting in the production of the insoluble form of $A\beta$, namely $A\beta_{40}$ and the more hydrophobic $A\beta_{42}$ (Serrano-Pozo et al., 2011). These insoluble $A\beta$ deposits clump together extracellularly to form oligomers and ultimately plaques (Serrano-Pozo et al., 2011).

The amyloid cascade hypothesis is a well known posit that places highly insoluble A β 42 at center-stage, claiming that the accumulation of A β 42 initiates the sequence of pathological events that ultimately leads to the development of AD (Hardy and Allsop, 1991; Hardy & Higgins, 1992). This was one of the most widely accepted hypotheses and the main driver of various drug development efforts as it explained why various risk factors such as mutations of the Amyloid Precursor Protein (APP), Presenilin (PSEN), Beta-Secretase 1 (BACE), and Apolipoprotein (APOE) as well as other diseases such as trisomy 21 and chronic traumatic encephalopathy, which increases A β 42 levels, predisposes one to develop AD (Serrano-Pozo et al., 2011; Zhang et al., 2011; Schellenberg, 1995; Corder et al., 1994; Farrer et al., 1997; Selkoe & Hardy, 2016; Zigman et al., 1996; Washington et al., 2016; Blennow et al., 2016).

However, there are major downfalls to this theory: firstly, studies and drugs that inhibit the accumulation of A β 42 did not find significant improvements to AD in humans; secondly, A β 42 has also been found in the post-mortem brain tissue of those who died healthily without AD; thirdly, there has not been any conclusive evidence demonstrating how the accumulation of A β 42 leads to the hyperphosphorylation of tau, or other related tauopathies; and finally, dense-core plaques, one of the two most common types of plaques found in AD, have been suggested to be produced by microglia and possess protective properties against diffuse plaques (Uddin et al., 2020; Jansen et al., 2015; Elobeid et al., 2016; Rodrigue et al., 2012; Huang et al., 2021).

Since then, various studies have shown that factors such as age, sex, socioeconomic status, education, sleep quality, and other environmental factors contribute to the development of AD (Riedel et al., 2016; Davis et al., 2020; Russ et al., 2013; Paradise et al., 2009; Ju et al., 2014; Iaccarino et al., 2021). These discoveries, along with the limited success of medical advances within the AD field, instigated researchers to consider the disease from new and broader perspectives, and as a multifactorial disorder (Iqbal and Grundke-Iqbal, 2010).

ALZHEIMER'S DISEASE RISK FACTORS

Age

Age is one of the greatest risk factors for AD, with the majority of AD cases found in patients ages 65 and older, and early onset in patients ages below 65 found in only 5-10% of all AD cases ("Early Onset Dementia, A National Challenge, A Future Crisis, Alzheimer's Association," 2006). Morbidity from AD increases exponentially after the age of 60, where 5.3% of people aged 65 to 74, 13.8% of people aged 75 to 84, and 34.6% of people aged 85 or older are estimated to have AD (Masters et al., 2015), making research on cortical aging imperative to a better understanding of AD itself. With the increase in the aging population, morbidity for AD is projected to increase to 152 million cases worldwide by 2050 according to the World Health Organization (WHO), making AD a pressing global issue.

Old, healthy brains share many similarities with the diseased brain. For example, despite being considered hallmarks of AD, NFTs and A β plaques were also found in the brains of healthy, aged people in population-based autopsy studies (Elobeid et al., 2016). Aged brains also have a diminished ability to sustain functional homeostatic environments which can cause increased reactive oxygen species and impairment of mitochondrial metabolism; two characteristic features of neurodegenerative diseases (Li et al., 2013;). Furthermore, microglia in the aged brain undergo morphological changes and show a similar decrease in functionality as seen in AD (Damani et al., 2011; Floden & Combs, 2011). The ubiquity of these findings in conjunction with increased morbidity for AD with age has led to two general hypotheses: the first considers AD as a form of accelerated aging, while the second claims that the characteristic pathogenesis of AD occurs in the majority of old brains, but some individuals possess compensatory coping mechanisms to maintain cognitive function (Toepper, 2017; Armstrong, 2019). Therefore, it would be beneficial to understand the differences between healthy aging and AD, and identification of potential factors that influence an individual's predisposition to AD could lead to new avenues for medical intervention.

Metabolic Dysfunction

The brain consumes approximately 20% of our body's entire oxygen intake to drive energy-expensive processes such as neuronal firing, gene expression, and structural changes at the synapses (Attwell & Laughlin, 2011). Healthy, mature neurons in the brain rely mainly on glucose for energy and produce ATP through the electron transport chain in the mitochondria (Mergenthaler et al., 2013), and this high energy dependency may render the brain highly susceptible to disease when energy metabolism is impaired.

Metabolic dysfunction is observed in AD with many studies reporting regional impairment of glucose uptake and metabolism in brain areas involved with memory and cognition (Mosconi, 2013; Mosconi et al., 2005; De Santi et al., 2001). Additionally, important mitochondrial processes that drive oxidative phosphorylation, such as the tricarboxylic acid cycle and the electron transport chain, were also compromised (Xu et al., 2016). The shift away from oxidative phosphorylation and adoption of a glycolytic signature is akin to the Warburg effect well known in the cancer field, and uncovering its implications with AD is at the forefront of ongoing research (Traxler et al., 2022).

Another relationship between AD and metabolic dysfunction is demonstrated by the two to threefold increase in the risk of developing AD in Type 2 Diabetes Mellitus (T2DM) patients, which occurs in around 50% of the patients (Leibson et al., 1997). Elderly T2DM patients who had experienced severe hypoglycemic episodes were more likely to develop dementia, and impairment in the insulin signaling pathway observed in AD brains has been linked to A β accumulation (Whitmer et al., 2009; Bedse et al., 2015). Therefore, although the exact mechanisms are still unknown, these findings demonstrate a distinct relationship between metabolic dysfunction and increased risk of AD.

Neuroinflammation

Inflammation is a biological process that protects the tissue against harmful stimuli. In the brain, one role of the acute inflammatory response is to clear neuronal debris and maintain homeostasis

(Galloway et al., 2019). Microglia, specialized macrophage and innate immune cells of the central nervous system, detect the deposition of A β and releases inflammatory cytokines to initiate clearance through phagocytosis; maintaining a balanced rate of A β production and clearance in cognitively normal participants (Cai et al., 2014; Simard et al., 2006; Mawuenyega et al., 2010). However, this ability to clear A β and prevent plaque formation is impaired in the AD brain (Ridolfi et al., 2013). A possible explanation may be the reduced ability of microglial receptors to bind to A β , or a failure to initiate phagocytotic machinery (Hickman et al., 2008; Fiala et al., 2005). Subsequently, the immune system attempts to counteract the persistence of A β deposits by recruiting more activated microglia, leading to the co-localized accumulation seen in post-mortem brain studies (Perlmutter et al., 1990). Simultaneously, as A β continue to accumulate, the microglia remain activated and adopt a pro-inflammatory M1 phenotype, increasing the production of reactive oxygen species and inflammatory cytokines, causing chronic inflammation (Hickman et al., 2008).

As previously stated, several studies of post-mortem AD brains reported sustained inflammation and the accumulation of microglia in areas with elevated amyloid plaques and NFTs (Perlmutter et al., 1990). Similar inflammatory phenotypes were conserved among other neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis among others, suggesting their significance in neurodegenerative diseases (Kinney et al., 2018). Moreover, non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen have been found to reduce AD disease pathology in a subset of patients (Jiang et al., 2008; Bolós et al., 2017). By inhibiting the production of pro-inflammatory cytokines such as NF κ B, anti-inflammatory drugs restrict the downstream pathways of these cytokines, alleviating both APP and A β levels, and reducing the risk of developing AD overall (Takada et al., 2004; Sambamurti et al., 2004; in t' Veld et al., 2001). However, the efficacy of NSAIDs and other anti-inflammatory drugs is an ongoing debate and their administration as treatment for AD has not been approved (Khan et al., 2019). All in all, these findings discuss the importance of inflammation and the role it plays in AD pathology.

ALZHEIMER'S DISEASE MODELING

Currently, post-mortem AD brains are the most accurate representation and gold standard for studying AD. However, the use of patient post-mortem tissue is accompanied by several challenges. For example, improper handling of the brain combined with long post-mortem delays can depreciate the quality of the samples (Pistollato et al., 2016). Furthermore, the freezing of brain tissue for storage can cause alterations in various functionalities such as a breakdown of transcriptional machinery and damage the molecular integrity of proteins (Pistollato et al., 2016). To combat this, various animals such as *Drosophila*, mice, and rats have been used to model the many aspects of AD.

One of the most common ways of modeling AD is by recapitulating tau and amyloid pathology (Phinney et al., 2003). This has been accomplished through various means such as the production of APOE4 and APP transgenic mice among many others (Drummond and Wisniewski, 2017; Chapman et al., 1999; Praticò et al., 2001). The modeling of other risk factors has also been utilized to study AD, for example, lipopolysaccharide (LPS), an endotoxin found on the outer membrane of gram-negative bacteria, has been used to induce neuroinflammation (Zakaria et al., 2017), and the subsection of animals to head injury has been used to model traumatic brain injury (Morganti-Kossmann, et al., 2010). Although much less common, *Drosophila* has also been used to study genetic mutations for the simplicity and low redundancy of their genome as well as the wide range of sophisticated molecular tools available (Bouleau & Tricoire, 2015).

However, there are many limitations to these animal models. Although they adequately imitate various aspects of AD pathology, none of them have succeeded in capturing the full range and complexity of the symptoms found in humans. Furthermore, the interspecific physiological differences restrict the extent of what we can reproduce in animals. For example, murine APOE is structurally and functionally distinct from human APOE, restricting the generalizability of mouse-study findings to humans (Tai et al., 2017). Most evidently, the lack of effective therapies and high rate of clinical failures despite various successful therapeutic attempts in animal models further highlights this discrepancy and rightly question

whether the findings in animal models are translatable to humans (Pistollato et al., 2016). Thus, a shift away from the current animal model-centric research paradigm is necessary (Pistollato et al., 2016).

Such advances in the modeling of AD were spearheaded by developments in cell reprogramming technology. In 2006, Takahashi and Yamanaka discovered that it was possible to reprogram somatic cells and force them to readopt pluripotency, producing induced pluripotent stem cells (iPSCs), through the administration of four crucial transcriptional factors, OCT3/4, SOX2, KLF4, and c-MYC, namely the “Yamanaka Factors” (Takahashi & Yamanaka, 2006). This breakthrough in cell reprogramming was revolutionary for regenerative medicine and opened up new avenues for disease modeling in various fields. In addition to its ability to differentiate into a wide variety of cell types, iPSCs retain most of the epigenetic memory of their donor, allowing them to accurately mimic human pathophysiology (Kim et al., 2010). For example, the use of iPSCs to study AD has allowed researchers to observe the functional effect of APOE4 in different cell types, a difficult feat as previously described (Meyer et al., 2019; Lin et al., 2018; Zhao et al., 2020).

In conjunction with this, advances in culturing technology added further dimensionality in the form of 3D organoids that increased the complexity of the models by simultaneously capturing diverse cell types and structural features of the human tissue. More importantly, the 3D nature of the organoids allows for the recapitulation of plaques and tangles, central components of AD (Gonzalez et al., 2018; Chen et al., 2021). However, a major limitation of using iPSCs in AD modeling is that the epigenetic signatures caused by age seem to be reverted with induced pluripotency (Mertens et al., 2015). Given that old age is one of the major risk factors of AD, direct conversion from fibroblasts into iNs is advantageous as the bypassing of the pluripotent stage allows the cells to retain the epigenetic information of the donor’s age (Torper et al., 2013; Mertens et al., 2015). Evidently, AD iNs successfully capture key aging-related features such as epigenetic erosion and loss of cell identity (Mertens et al., 2015). Although various challenges have arisen in the modeling of AD, significant advancements have made capturing the complex human-relevant physiology of AD possible, enabling an improved means of investigating this neurodegenerative disease.

CANDIDATE TRANSCRIPTION FACTORS

Detailed transcriptomic analysis of post-mortem AD brains discovered a gene expression signature unique to AD neurons (Mathys et al., 2019; Blalock et al., 2011). Previous research in our lab compared AD patient-derived iNs to age-matched controls, identifying over 700 highly significant differentially expressed genes (DEGs), and discovering an age-dependent enrichment of dedifferentiation-related pathways characteristic of AD (Mertens et al., 2021). For example, AD iNs were observed to restrict the expression of genes related to mature neuron function while highly upregulating glycolytic and transformation-associated genes, indicating a loss of neuronal identity and an adoption of a hypo-mature cellular program that could cause hallmark features of AD such as synaptic degeneration (Mertens et al., 2021; Koffie et al., 2011). YY1, RUNX1, NFIB, and PPAR γ emerged as some of the most significantly upregulated transcription factors with important developmental and cancer-associated roles that might contribute to the return-to-immaturity in AD neurons (Jiang et al., 2008; Denny et al., 2016; Fukui et al., 2018; Chen et al., 2019). As transcription factors, these proteins possess the ability to regulate the expression of other genes, often leading to phenotypic changes, making them attractive candidates for investigation. Further discussion of the current relevant knowledge surrounding these four TFs is detailed below.

YY1

Yin Yang 1 (YY1) is a ubiquitously expressed zinc-finger transcription factor known for its ability to act as both a repressor and activator of various genes and has been known to have a role in brain development and regulation of mitochondrial bioenergetics (Hyde-DeRuyscher et al., 1995). To regulate gene expression, YY1 forms heterodimers that bind directly to the 5'-CCGCCATNTT-3' sequence motif found on enhancers and promoters through its zinc-finger C-terminal domain (Hyde-DeRuyscher et al., 1995; Yant et al., 1995). It then recruits chromatin modifiers such as the polycomb complex, histone deacetylase (HDAC), and histone acetyltransferase (HAT) to repress and activate genes respectively (Yao

et al., 2001). YY1 can also interact with cohesin and condensin through its REPO domain to affect gene transcription through the 3D remodeling of the chromatin by forming DNA loops that mediate long distance promoter-enhancer interactions in T-helper cells and many other mammalian cells (Pan et al., 2013). This duality of its function is what eventually led to the name “Yin Yang” (Shi et al., 1991).

Functionally, YY1 has a well-established role during development and neuronal differentiation, and its expression peaks during the early stages and depreciates as development progresses into the late stages (Dong et al., 2020). YY1’s role in the neuron is highly context-specific and differs as the cell progresses through each stage. For example, YY1 maintains the pluripotency of neural progenitor cells (NPC) by regulating the expression of *WNT1*, a component of a crucial developmental signaling pathway during midbrain development, and forms NPC-specific promoter-enhancer looping interactions through the reorganization of the 3D genomic architecture (Dong et al., 2020; Beagan et al., 2017). During differentiation, YY1 controls mitochondrial bioenergetics, such that a knockdown of YY1 in developing mice led to a significant downregulation of mitochondrial gene expression, basal oxygen consumption rates, and subsequently, restriction of differentiation into mature neurons (Zurkirchen et al., 2019). Pathway enrichment and genomic analyses also revealed that several metabolic pathways were disturbed upon the knockdown of YY1, including oxidative phosphorylation, glucose metabolism, and the TCA cycle during neural crest development (Varum et al., 2019).

However, the YY1-regulated metabolic shift appears to act in a context-specific manner. During injury-induced regeneration in satellite cells, YY1 facilitates the metabolic reprogramming from oxidative phosphorylation back to glycolysis. To accomplish this, YY1 first binds to and represses the expression of mitochondrial genes, restricting oxidative phosphorylation (Chen et al., 2019). Following this gene repression, glycolytic genes such as LDHA are indirectly activated through the stabilization of the HIF1 α protein (Chen et al., 2019). Together, these processes promote the switch to glycolysis and are required for stem cell proliferation during regeneration. Thus, YY1 seems to have a dual role in the regulation of metabolic programs by promoting mitochondrial expression of oxidative phosphorylation-related genes during development, while also promoting the glycolytic pathway that restricts differentiation during

tissue regeneration. All in all, these findings have identified YY1 as a key driver of brain development and regulator of metabolic pathways. However, the role of YY1 in aged neurons and its relevance to AD remains a mystery due to the duality and high context-specificity of its nature, thus warranting further research.

RUNX1

Runt-Related Transcription Factor-1 (RUNX1) is a member of the core-binding factor family which modulates cell proliferation, differentiation, and survival in multiple systems (Marmigère et al., 2006; Chuang et al., 2013; Ito & Miyazono, 2003; Burns et al., 2005). RUNX1 has highly conserved dual promoters: a distal P1 and proximal P2 promoter, which allows for high tissue specificity (Sroczynska et al., 2009). RUNX1 is distinguishable by its DNA-binding “Runt” domain at the N-terminus and a transcription activation domain at its C-terminus (Fukui et al., 2018). Initially found in DNA obtained from an acute myeloid leukemia patient, RUNX1 is also known as Acute Myeloid Leukaemia 1 protein (AML1) for its involvement with hematological malignancies. (Miyoshi et al., 1991)

Known for its role as a hematopoietic master regulator, RUNX1 regulates the expression of hematopoietic genes required for the transition of hemogenic endothelium cells into hematopoietic progenitors (Chuang et al., 2013). RUNX1 levels increase during the G1 to S transition and remain elevated at the G2/M phase in hematopoietic cells and its absence leads to a complete lack of hematopoietic stem cells (HSCs) in the aortic epithelium (Bernadin-Fried et al., 2004; Okuda et al., 1996).

RUNX1 retains its role as a regulator of differentiation in the CNS, promoting the neural lineage during neurogenesis (Marmigère et al., 2006). In the mouse hippocampus, the learning and memory formation hub of the brain, RUNX1 expression decreases as neuronal precursor cells differentiate into neurons and accumulate neuronal marker genes like *Neurog2* and *Lmo3* (Fukui et al., 2018). RUNX1 is then regulated by hippocampal precursor cell markers such as TGF- β 1 and Notch1 and further depreciates to low levels in the adult mouse brain (Ito & Miyazono, 2003; Burns et al., 2005). RUNX1 expression is

also found to be significantly elevated following cortical injury and led to an increase in microglial activation and neural stem cells proliferation in the hippocampal dentate gyrus, highlighting RUNX1's potential reparative roles following injury (Logan et al., 2013; Eriksson et al., 1998).

RUNX1 activity has also been linked to the immune system and inflammation, although mechanistic variations exist in a context-specific manner. In hematopoietic cells, RUNX1 attenuates the NF- κ B signaling pathway by inhibiting I κ B Kinase (IKK) (Nakagawa et al., 2011; Bellissimo et al., 2020), while in macrophages RUNX1 enhances NF- κ B signaling which promotes the production of IL-1 β and IL-6 (Luo et al., 2016). Additionally, altered functionality of RUNX1 has been found in patients with autoimmune diseases such as rheumatoid arthritis and lupus, diseases with high predisposition for AD (Alarcón-Riquelme et al., 2004).

Correspondingly, in the context of AD, SNPs within the RUNX1 gene have been linked to peripheral levels of brain-derived neurotrophic factor (BDNF), a potential marker for AD (Li et al., 2020). Potential binding sites for RUNX1 have also been found in highly conserved sequences of BDNF in Zebrafish, raising the possibility of RUNX1 indirectly regulating hippocampal long term potentiation, learning, and memory (Heinrich & Pagtakhan, 2004). Additionally, genomic analysis of down syndrome patients found RUNX1 SNPs to be strongly associated with dementia of AD and increased A β levels (Patel et al., 2011). Considering RUNX1's diverse functions in the regulation of differentiation and inflammation, it is reasonable to speculate that RUNX1 may contribute to the pathogenesis of AD, thus further study of RUNX1 would be beneficial.

PPAR γ

Peroxisome Proliferator Activated Receptor Gamma (PPAR γ), a member of the peroxisome proliferator-activated receptor (PPAR) family, is a nuclear receptor that is highly involved in adipogenesis, energy metabolism, and anti-inflammatory responses (Qi et al., 2000). PPAR γ has six protein domains including a highly conserved DNA-binding domain, ligand binding domain, and two zinc

fingers that permit its performance as a transcriptional regulator (Qi et al., 2000). There are two PPAR γ isoforms in mammals, PPAR γ 1 and PPAR γ 2, and as the name suggests, PPAR γ is activated by peroxisome proliferators such as long-chain fatty acids, prostaglandins, and synthetic drugs (Zhu et al., 1995). Once activated, PPAR γ forms heterodimers with retinoid X receptors and binds to DR-1 motifs (direct hexanucleotide repeats) of the cis-acting peroxisome proliferator response elements (PPREs) to influence the transcription of various genes (Tontonoz et al., 1994).

One well known function of PPAR γ is its role as the master regulator of adipogenesis (Moldes et al., 2003). During adipogenesis, PPAR γ drives adipocyte differentiation by blocking the Wnt signaling pathway through the degradation of β -catenin (Moldes et al., 2003), and promoting the expression of adipocyte differentiation genes (Rosen et al., 1999; Siersbaek et al., 2010). Given the metabolic importance of adipose tissue, dysfunction of PPAR γ can lead to increased insulin resistance and ultimately, metabolic diseases such as lipodystrophy, obesity, and diabetes (Barroso et al., 1999; Guilherme et al., 2008). In the brain, PPAR γ is expressed in neurons in the hypothalamus, the area involved in energy homeostasis, regulation of food intake, and weight gain (Liu et al., 2015). The use of synthetic PPAR γ agonists such as thiazolidinediones is effective against insulin sensitivity and the latter has been widely used as a treatment for diabetic patients (Ament et al., 2012).

PPAR γ has also emerged as a key regulator of inflammation in several cell lines such as macrophages where the function of IL-4, an anti-inflammatory cytokine, is dependent on PPAR γ (Glass & Saijo, 2010; Odegaard et al., 2007). Similarly, the activation of PPAR γ can reduce the production of inflammatory cytokines such as TNF α and NF- κ B which prevents insulin resistance (Olefsky & Glass, 2010). The anti-inflammatory effect of PPAR γ is responsible for the prevention of microglial activation and A β plaque formation (Shie et al., 2009). It was previously demonstrated that NSAIDs that activate PPAR γ inhibit cyclooxygenases to repress inflammation, and high doses of PPAR γ agonists such as rosiglitazone can rescue the accumulation of A β in the brain (in t' Veld et al., 2001, Jiang et al., 2008). However, the relevance of PPAR γ to AD is still controversial as different clinical studies report large discrepancies in the efficacy of PPAR γ agonists in alleviating AD-related symptoms (Khan et al., 2019).

All in all, the therapeutic potential of PPAR γ agonists in addition to their high expression levels in the hippocampus makes PPAR γ another candidate worth investigating.

NFIB

Nuclear Factor 1 B-type (NFIB) is a part of the Nuclear Factor 1 (NFI) family that controls gene expression by binding to the 5'-TTGGCNNNNNGCCAA-3' palindromic sequence (Harris et al., 2015). Originally known for its essential role in adenovirus replication, NFIB has also been associated with lung and brain development, and metastasis of various cancers (Steele-Perkins et al., 2005). In humans, NFIB is highly expressed in multiple organs during development and has been found to operate in a context-specific manner, promoting cell differentiation during development but stem-cell renewal in adult tissues (Denny et al., 2016; Harris et al., 2015). For example, an NFIB knockout in prenatal mice disrupts lung development and leads to severe hypoplasia, a trait that becomes lethal once the mouse is born and depends on its own lungs for respiration (Gründer et al., 2002). Similarly, NFIB is essential for normal brain development and seems to cooperate with NFIA to regulate growth and differentiation of both the forebrain and hindbrain structures (Steele-Perkins et al., 2005). NFIB null mice experienced delayed glial and neuronal differentiation, and eventually developed macrocephaly, an enlargement of the brain and head, and intellectual disability (Schanze et al., 2018). Other studies revealed that NFIB regulates the expression of genes related to neuronal migration, axonal projection, and cell cycle progression (Betancourt et al., 2014). Consistent with this, NFIB is also found to be expressed by neuroblasts in stem cell niches of the adult CNS (Plachez et al., 2012).

NFIB is also often described as an oncogene, promoting tumorigenesis in small-cell lung cancers (SCLC), melanomas, and most breast cancers among others (Kim et al., 2018; Cheng et al., 2021; Chen et al., 2020). In SCLC cells, NFIB causes a widespread increase in chromatin accessibility by competitively binding to the DNA, preventing its interaction with nucleosomes (Denny et al., 2016). This hyper-accessible chromatin landscape permits the expression of various genes, including those that

promote metastasis (Denny et al., 2016). Its potency for epigenetic erosion is reflected by the finding that almost all metastatic high-grade neuroendocrine lung tumors overexpress NFIB (Semenova et al., 2016). However, NFIB has also recently been identified as a tumor suppressor in glioblastoma and triple-negative breast cancers, suggesting a dual cancer-specific role (Stringer et al., 2016; Liu et al., 2019). A similar widespread increase in chromatin accessibility, akin to undifferentiated neurons and cancer cells, as well as an upregulation of NFIB, was identified in AD iNs (Mertens et al., 2021). Thus, further exploration of NFIB's role in the brain would be beneficial to understanding its potential contribution to AD development.

BASIS OF THIS PROJECT

YY1, RUNX1, NFIB, and PPAR γ were previously found to be highly expressed in AD neurons and regulate various processes, found to be disrupted in AD, in various cell types (Mertens et al., 2021; Kim et al., 2018; Shie et al., 2009; Heinrich & Pagtakhan, 2004; Chen et al., 2019). However, the current lack of research on their function in the context of old age limits our ability to ascertain their role in AD. Therefore, to investigate the contribution of each TF to AD, we questioned their individual ability to induce similar biological dysfunctions phenotypic of AD in aged neurons. We profiled the TF-induced transcriptomic changes in iNs derived from direct reprogramming of human fibroblasts into neurons, and tested the translatability of these gene expression changes via functional assays.

The work presented here has been conducted under the supervision of my supervisors Dr. Raffaella Lucciola, Ph.D. and Professor Rusty Fred Gage. Conceptualization, methodology, formal analysis, investigation, resources, data curation, review, and editing, Dr. Raffaella Lucciola, Ph.D. and Professor Rusty Fred Gage.

METHODS

Subjects:

Fibroblast donors of this project were participants at the Shiley-Marcos Alzheimer's Disease Research Center (ADRC) at the University of California, San Diego (UCSD) (Fig. 1C). Written consent was collected from all donors and all procedures were approved by local human subjects committees. Clinical assessments including detailed neuropsychological testing and brain imaging (MRI) were performed as part of the ADRC study and "healthy" subjects demonstrated an explicitly non-demented clinical image. Punch skin biopsies were obtained at the Altman Clinical & Translational Research Institute (ACTRI) at UCSD, and fibroblast derivation was performed by standard procedures in serum-containing media. Subjects were followed for two years post punch biopsy to ensure no evidence of cognitive decline.

Cell culture and iN Conversion:

Cell culture and iN conversion was done following protocols from Mertens et al (2021). Fibroblasts from female donors over 65 years of age (Fig. 1C) previously transduced with the UNA construct (pLVXUbc-rtTA-Ngn2:2A:Ascl1) were thawed from N₂(l) storage and cultured in TFM-P medium: DMEM + GlutaMAX (ThermoFisher cat#10569-010) containing 15% tetracycline-free fetal bovine serum (Omega Scientific cat#FB-15), 1% MEM NEAA (ThermoFisher cat#11140050), and 1µg/mL puromycin (Gibco cat#A11138-03). Once full confluency was reached, fibroblasts were trypsinized (Gibco cat#12604-013) and pooled onto Poly-L-Ornithine and Laminin-coated (PORN-L) dishes at a ratio of 3:1 to increase cell density for iN conversion. 24 hours post pooling, culture medium was switched to neural conversion (NK) medium to induce conversion from fibroblast to neuron (Fig. 1A-B). The switch from TFM-P to NK medium marks day 0 of conversion. NK medium: DMEM:F12+GlutaMAX/Neurobasal A (1:1) (ThermoFisher cat#10565-018, ThermoFisher cat#10888-022), 1x B27 supplement (ThermoFisher cat#12587010), 1x N2 supplement (ThermoFisher

cat#A1370701), 1 μ g/mL Laminin (Gibco cat#23017015), 400 μ g/mL dibutyl-cyclic-cAMP (TOCRIS cat#1141), 2 μ g/mL doxycycline (STEMCELL Technologies cat#72742), 150ng/mL human recombinant Noggin (Proteintech cat#HZ-1118), 0.5 μ M LDN-193189 (AdooQ cat#A11478), 0.5 μ M A83-01 (AdooQ cat#A12358-50), 3 μ M CHIR-99021 (AdooQ cat#A10199-100), 3 μ M Forskolin (AdooQ cat#A12917-100), 10 μ M SB-431542 (StemRD cat#SB-050). Fresh medium is replenished to both fibroblasts and iNs every Monday, Wednesday, and Friday.

Overexpression of YY1, NFIB, RUNX1, and PPAR γ

Competent bacteria (E.coli) transfected with the construct: pLV[Exp]-TRE3G>hTF-hPGK>EGFP (Fig. 1D) were grown in Luria Broth (LB) containing ampicillin overnight with shaking at 37°C. Plasmid was isolated following MaxiPrep protocol (Life Tech, Invitrogen K2100-07) and evaluated for quality via restriction digest. Lentiviral vectors were produced by the GT3 Core Facility of the Salk Institute and transduced into iNs via media for 24 hours. Overexpression of TFs was checked by identification of EGFP+ cells under fluorescence microscopy eight days post transduction (Fig. 1E) and subsequently selected by fluorescence activated cell sorting (FACS).

Culture Dish Treatment with Poly-L-Ornithine and Laminin (PORN-L)

Culture dishes were coated with PORN-L prior to pooling and iN conversion to prevent loss of cells via detachment. To coat, 10 μ g/mL Poly-L-Ornithine (Sigma cat#P3655) solution in sterile deionized (DI) water was added to the plates, ensuring complete coverage of growth surface area, and incubated overnight in a sterile environment at room temperature. Plates were washed twice with sterile DI water and a second coating of 5 μ g/mL Laminin (Invitrogen, Natural Mouse, cat#23017-015) solution in DPBS was added, again, ensuring full coverage, and incubated overnight in a sterile environment at room temperature. The Laminin solution was aspirated before use with no washing. For long-term storage, plates were wrapped with plastic wrap and stored on a level surface at -20°C.

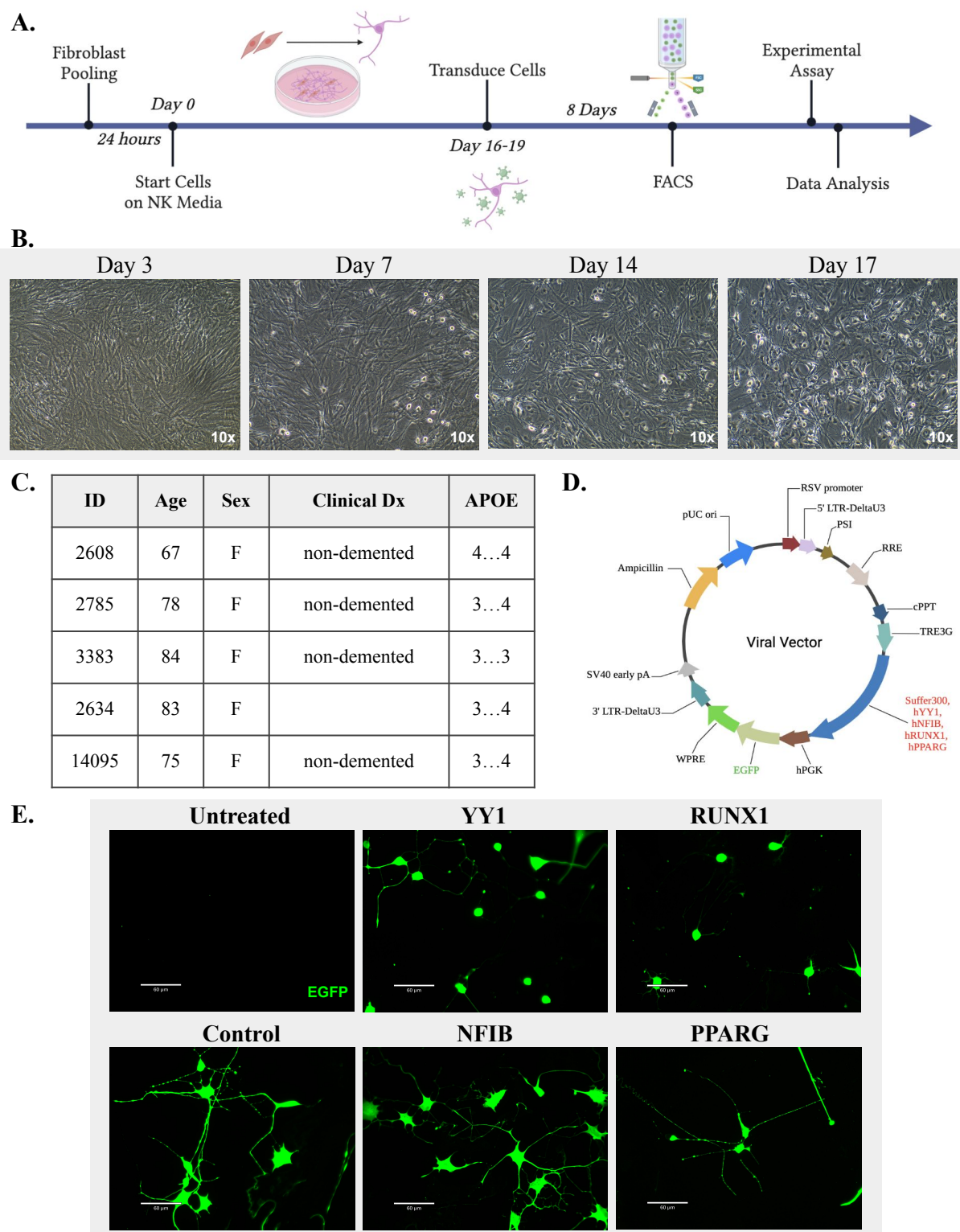


Figure 1. Production of Induced Neuron and Transcription Factor Overexpression. (A) Schematic of the general timeline for each experiment created with BioRender. (B) Light microscope images of cells during conversion from fibroblast to induced neuron (iN) on days 3, 7, 14, and 17 days of culture in NK media (10x). (C) Donor ID and information. (D) Plasmid map for viral vector used to overexpress the transcription factors. Red = variable region. (E) Immunofluorescence images of iNs 8 days post transduction.

Fluorescence-Activated Cell Sorting:

Cells were detached with TrypLE (Gibco cat#12604-013) containing 0.1% DNase (Thermo Scientific cat#01290377) to prevent clumping and washed with cyto buffer (800 mL of distilled water containing 200 mL DPBS without Mg^{2+}/Ca^{2+} ; 43.25g Myo-Inositol, MP Biomedicals cat#194688; and 5g Polyvinyl Alcohol Sigma-Aldrich cat#p8136-250G) containing 5% of 0.1% KOSR (Lifetech cat#10828-028). Cells were then stained with PSA-NCAM directly conjugated to APC (Miltenyi Biotec cat#130-120-437, 1:100) in cyto buffer + 5% KOSR for 45 minutes at 4°C, washed with cold cyto buffer + 5% KOSR, resuspended in 300µL sorting buffer (cyto buffer containing 5mM EDTA, 1:500 0.1% DNase, and DAPI), and filtered using a 40-µm cell strainer. Fluorescence-activated cell sorting (FACS) was utilized to isolate iNs overexpressing the transcription factor by selecting for PSA-NCAM:APC and EGFP double positive cells from DAPI negative live cells 8 days post transduction. Untreated iNs stained with the isotype control antibody (Miltenyi Biotec, mouse IgM APC clone-1S5-2004, cat#130-122-975) were used as the PSA-NCAM:APC negative population to set the gate threshold during sorting.

For RNA isolation, cells were sorted into Trizol-LS (Ambion cat#10296010), while for replating, cells were sorted into NK+++: NK media containing ROCK inhibitor (AdooQ cat#A11001, 1:1000), Z-VAD (UBPBio cat#F7111, 1:1000), and penicillin-streptomycin (Gibco cat#15140-122, 1:100).

Sample preparation was performed by Yatavee Vajaphattana, Dr. Raffaella Lucciola, and FACS was performed by Michelle Liem and Mikayla Marrin from the Flow Cytometry Core at the Salk Institute for Biological Sciences.

RNA-seq and Analysis:

To extract RNA, the protocol for RNA clean and concentrator (Zymo Research cat#R10414) was followed and sequencing was performed by the next-generation sequencing core at the Salk Institute.

RNA-seq analysis: Read trimming (Trim Galore), genome alignment (STAR), abundance estimation (Salmon), differential expression analysis (DESeq2), functional enrichment analysis (overrepresentation analysis and gene set enrichment analysis).

Cell culture was performed by Yatavee Vajaphattana, RNA-sequencing was performed by the Next Generation Sequencing Core at the Salk Institute for Biological Sciences, and bioinformatic analysis of RNA-seq data was performed by Mike Cuoco, Lukas Karbacher, Dr. Thais Sabedot, and Dr. Raffaella Lucciola.

pPKM2 Immunostaining:

iNs were transduced with lentivirus for 24 hours to overexpress the TF of interest on day 16, and successfully transduced mature iNs were isolated via FACS on day 21 by selecting for PSA-NCAM/EGFP double positive cells (Fig. 1A). Sorted iNs were replated onto PORN-L coated 96 well Ibidi at a density of approximately 50,000 cells per well in NK+++ and incubated overnight. Media change was performed the following day using NK containing anti-anti (Gibco cat#15240-096). The supernatant was collected for lactate quantification 48 hours after media change and stored at -80°C.

For pPKM2 immunostaining, cells were fixed with 4% PFA for 20 minutes at room temperature and washed 3 x 15 minutes with TBS, followed by a 1-hr block with TBS containing 10% horse serum and 0.1% Triton X-100. Cells were stained with primary antibodies (pPKM2 Ser35 rabbit, SAB Biotherapeutics cat#11456, 1:500; TUJ1 mouse, Biolegend, lot#B299272, 1:500; EGFP chicken, Ares Lab, lot#GFP3717982) overnight at 4°C. Following DNA staining with DAPI (1/10,000; Sigma-Aldrich) and TBS washing, secondary antibodies (Cy3 anti-rabbit, 1:500; Cy5 anti-mouse, 1:500; Alexa Fluor 488 anti-chicken, 1:500) were incubated for 2 hours at room temperature in darkness. After washing, slides were mounted in PVA-DAPCO (Sigma Aldrich), and fluorescence images were taken using a standard fluorescence microscope.

Cell culture, treatment, and collection of data were performed by Yatavee Vajaphattana and Lukas Karbacher. Analysis of pPKM2 nuclear translocation and lactate quantification was performed by Dr. Larissa Traxler and Dr. Raffaella Lucciola.

Morphology Reconstruction:

Fully confluent fibroblasts were pooled directly into PORN-L coated 4-well Ibidi at a ratio of 3:1 and switched to NK medium 24 hours post pooling. iNs were transduced with lentivirus to overexpress our TF of interest on day 18, and fixed 8 days post transduction. Fixed iNs were stained with either stain 1 primary antibodies (MAP2a + 2b mouse mono, Sigma-Aldrich, 1:200; Synapsin1 rabbit, Calbiochem, 1:100; EGFP chicken, Ares Lab, 1:2000) and secondary antibodies (Cy5 anti-mouse, 1:500; Cy3 anti-rabbit, 1:500; Alexa Fluor 488 anti-chicken, 1:500); or stain 2 primary antibodies (PSD95 rabbit, Invitrogen, 1:250; TUJ mouse, BioLegend, 1:500; EGFP chicken; Ares Lab; 1:2000) and secondary antibodies (Cy3 anti-rabbit, 1:500; Cy5 anti-mouse, 1:500; Alexa Fluor 488 anti-chicken, 1:500) for 2 hours.

Immunofluorescence images were taken on standard fluorescence microscopes at a magnitude of 20x. Composite images were produced in FIJI (Fig. 2A-B) (Schindelin et al., 2012), and neuron reconstruction was performed using the Neurolucida software (MBF Bioscience) by manually tracing EGFP/TUJ or EGFP/MAP2a+2b double positive neurons. Tracings were then analyzed for average dendritic length using the built-in branched structure analysis in Neurolucida Explorer (MBF Bioscience), and statistical analysis was performed in GraphPad Prism.

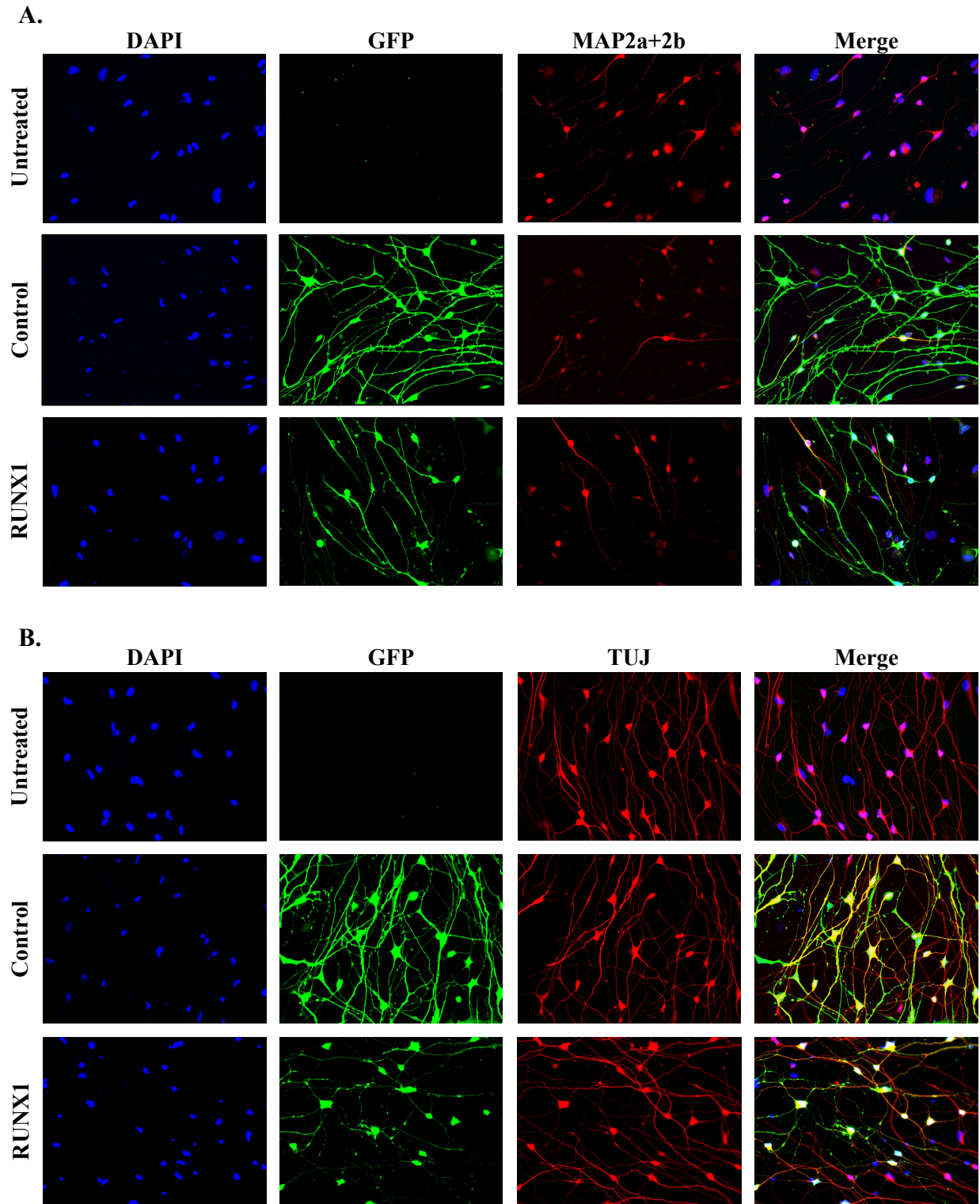


Figure 2. Fluorescence Imaging for Morphological Reconstruction of Neurons. Representative fluorescence images (20x) of unsorted untreated, control, and RUNX1-overexpressing induced neurons (iNs) from patient 2785 labeled with DAPI (blue), EGFP (green), and **(A)** MAP2a+2b (red) in “Stain 1” or **(B)** TUJ (red) in “Stain 2”. Composite images were produced by merging each colour channel in FIJI.

RESULTS

Transcriptional Effect of YY1, RUNX1, NFIB, and PPAR γ in Old Induced Neurons

YY1, RUNX1, NFIB, and PPAR γ have been found to regulate various biological processes that are disrupted in AD in various cell types, but, to the best of our knowledge, there has been no record of their roles in old human neurons. To characterize the effect of each TF in aged neurons, we individually overexpressed each TF in iNs derived from old, healthy, donors and profiled the changes in gene expression via bulk RNA-seq.

To implement quality control on our data, we performed dimensionality reduction using principal component analysis (PCA) and plotted the principal components based on the highest representation of variation (Fig. 3A-B). The highly significant increase in expression of each TF (YY1 Log₂FC = 7.14, padj = 7.34E-133; RUNX1 Log₂FC = 11.5, padj = 1.63E-73; NFIB Log₂FC = 10.8, padj = 3.04E-33; PPAR γ Log₂FC = 11.1, padj = 2.68E-28) indicates a successful overexpression of our TFs of interest (Fig. 3C, 4C), and the PCAs revealed that, of them three patient samples, cells derived from individual 14095 clustered differently to the other two indicating a possible biological difference between this individual and the others (Fig. 3A, C; PC2vsPC3, PC2vsPC5, PC3vsPC4). The PCA plots also revealed that RUNX1 and YY1 cluster differently from other conditions on different occasions, indicating a notable difference in the gene expression pattern caused by RUNX1 and YY1 overexpression (Fig. 3A). Interestingly, YY1 was found to be downregulated in other conditions (Fig. 3C), suggesting that YY1 may be a common downstream effector or mediator of the other TFs and may be worth further investigation.

DGE analysis revealed that YY1 and RUNX1 have the strongest effect on gene expression in aged neurons overall, causing more genes to be differentially expressed relative to control than NFIB and PPAR γ (Fig. 4A). YY1 and RUNX1 also shared more highly significant

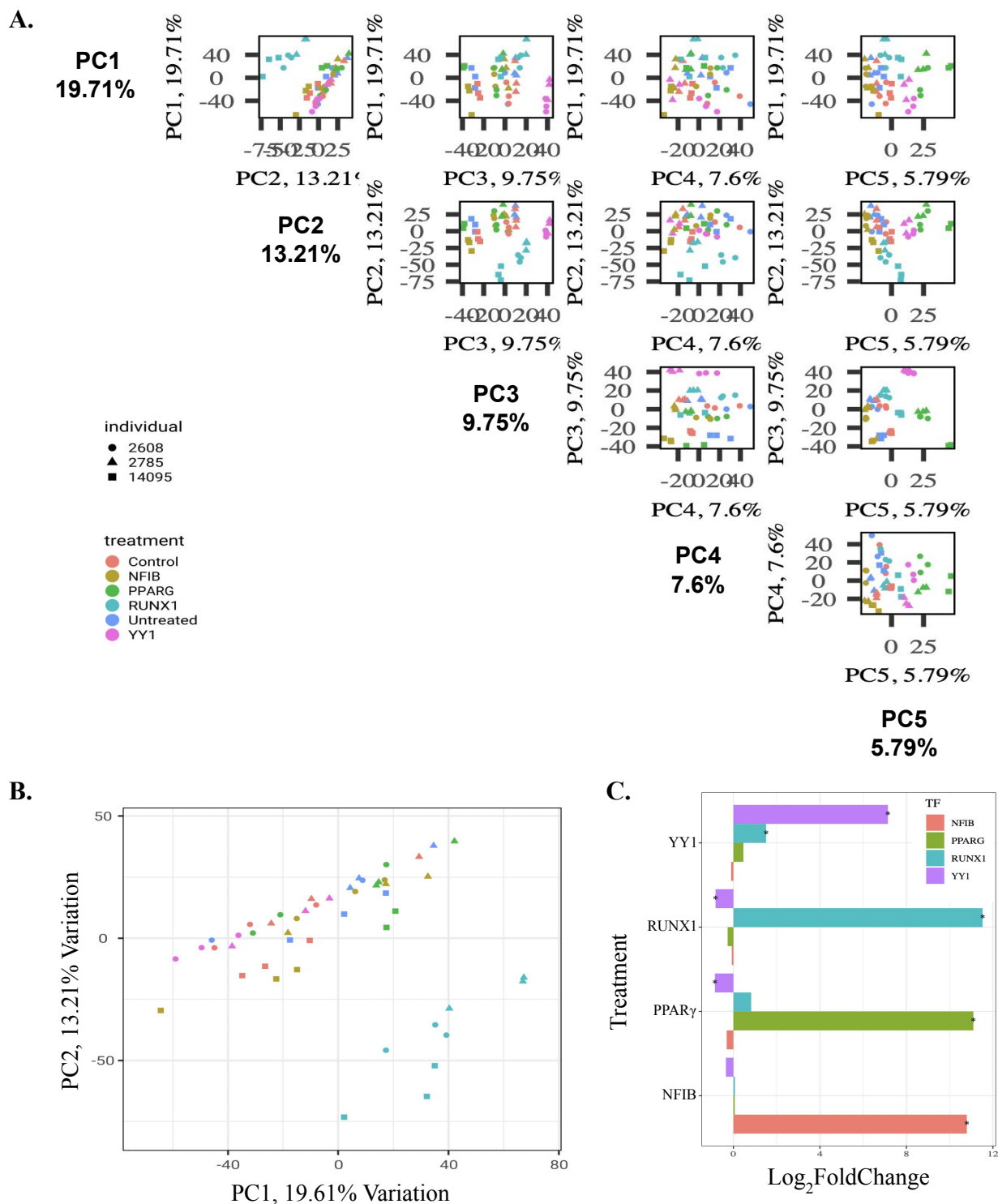
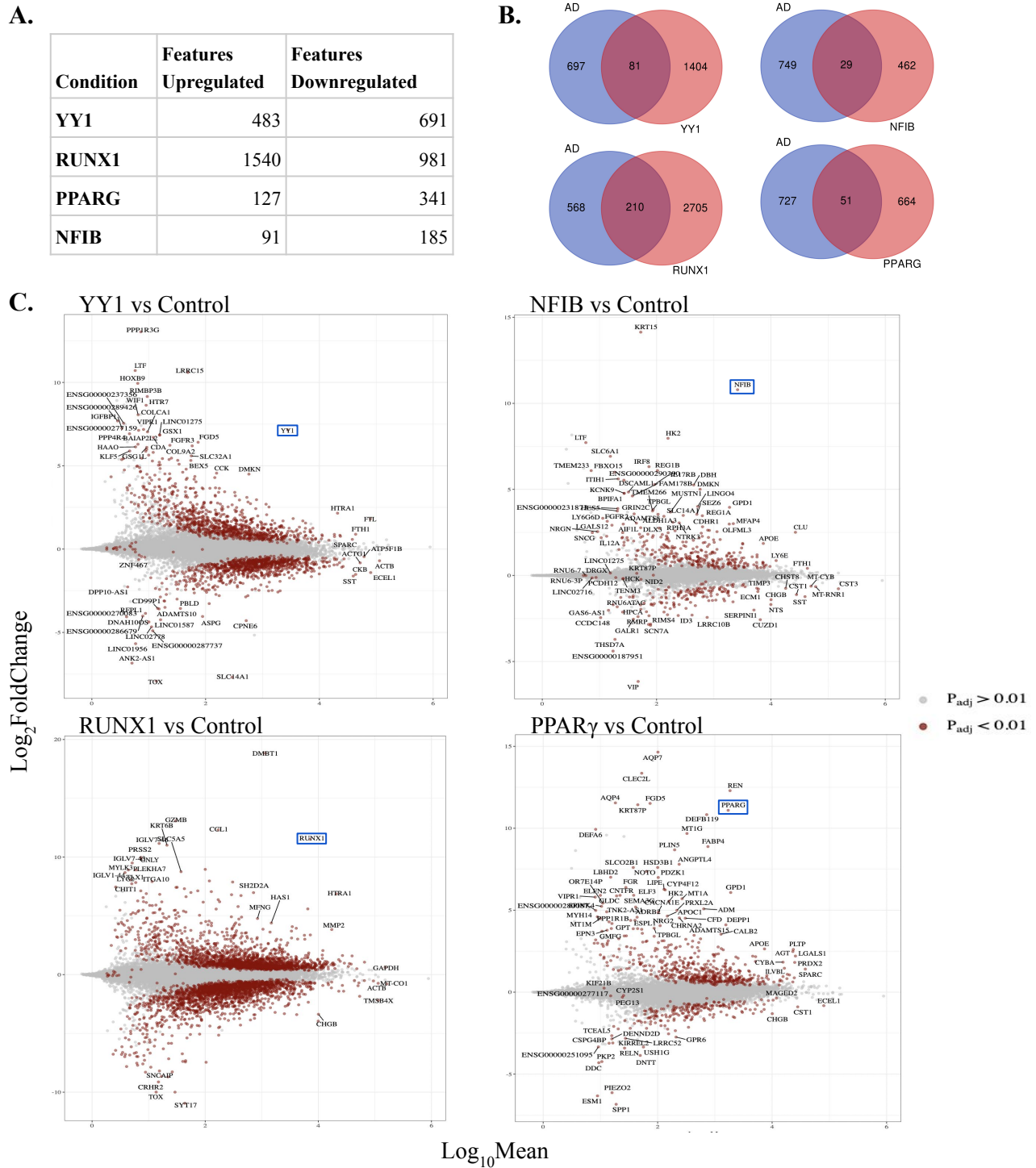


Figure 3. Quality Control and Exploration of Association Between Samples in Various Dimensions. (A) PCA plots including PCs from PC1(19.71%) to PC5 (5.79%) captures variations in clustering among PC pairs. **(B)** Principal component analysis (PCA) of RNAseq data from all three biological replicates (individuals), PC1 vs PC2. **(C)** Gene expression changes, shown as Log₂FC, of the indicated TFs when each TF is overexpressed. Statistical significance was assessed using One-way ANOVA $p < 0.05$.



DEG with AD iNs from the Mertens et al (2021) dataset than NFIB and PPAR γ , but RUNX1 had significantly more similarities to AD than any other condition (Fig. 4B).

YY1 Induces a Metabolic Shift From Oxidative Phosphorylation to Glycolysis in Neurons

To construe biological relevance from the DEGs, we performed gene set enrichment analysis (GSEA) based on the results of our DE analysis. When examined individually, YY1 exhibited a strong influence on neuronal metabolic regulation, downregulating various terms related to metabolism (Fig. 5A). Of the downregulated Gene Ontology (GO) terms, all top 10 most negatively enriched terms were related to oxidative phosphorylation and the mitochondria (Fig. 5A), and concurrently, we observed a clear depletion of the important hallmark term oxidative phosphorylation (Fig. 5B). This data is consistent with the observation that AD neurons abandon the electron transport chain-driven metabolic program typical of mature neurons, and undergo an aberrant switch to glycolysis, known as the Warburg-like effect (Mertens et al., 2021; Traxler et al., 2022). However, no significant changes in the expression of canonical glycolytic genes such as lactate dehydrogenase (*LDHA*; Log₂FC = 0.23, padj = 0.51) and hexokinase 2 (*HK2*; Log₂FC = 0.29, padj = 0.04) nor enrichment of the ‘glycolysis’ hallmark term itself were observed in our dataset (Fig. 5B).

A recent study reported a neuron-specific increase in pathological splicing of pyruvate kinase M (PKM), a gene associated with glycolysis and lactate production, to produce its cancer-associated PKM2 isoform in AD iNs (Traxler et al., 2022; Yang et al., 2013). The accumulation of PKM2 isoform is accompanied by increased translocation of phosphorylated PKM2 (pPKM2 Ser37) to the nucleus where pPKM2 acts as a protein kinase to facilitate HIF1 α , STAT3, and β -catenin activity, inducing the Warburg-like metabolic shift seen in AD iNs (Traxler et al., 2022; Mertens et al., 2021). To examine the translatability of the changes in gene expression to functional phenotype, we further investigated the effects of YY1 on metabolism by measuring pPKM2 nuclear translocation and quantifying lactate production using colorimetric lactate assays. We observed a significantly higher proportion of pPKM2 localized in the nucleus in YY1-overexpressing iNs compared to control and all other conditions (Fig.5C).

Interestingly, RUNX1 also increased nuclear pPKM2 in iNs, although to a lesser extent (Fig. 5C). Although data from our lactate analysis is still being validated, the initial results demonstrate an increase in lactate production in YY1-overexpressing iNs, indicative of an increase in glycolysis despite the aforementioned lack of significant changes in glycolytic gene expression detected in the RNA-seq (Fig. 5B). Together, these findings show that YY1 can initiate metabolic reprogramming in healthy, aged iNs and could contribute to the metabolic dysregulation in AD patients by repressing the expression of oxidative phosphorylation-related genes and facilitating pPKM2 translocation into the nucleus.

RUNX1 Restricts Neuronal Marker Expression and Alters Neuronal Morphology

GO enrichment analysis revealed that RUNX1-overexpressing iNs encompass a wide and highly significant depletion of terms related to neuronal development, structure, and synaptic signaling similar to AD iNs throughout all three domains: biological process (BP), cellular compartment (CC), and molecular function (MF) (Fig. 6A). Of the highly significant downregulated genes, the majority were closely related to brain development (Fig. 6B), suggesting a possible link to the cellular dedifferentiation seen in AD (Mertens et al., 2021). Axonal guidance-related genes were also downregulated (Fig. 6F).

Neuronal morphology is a good indicator of health and function, and the loss of synaptic connections and reduction in neuronal complexity is characteristic of AD neurons (Peng et al., 2021; Udvary et al., 2022; Mertens et al., 2021). We further questioned whether the RUNX1-induced restriction of neuronal gene expression would affect morphology and reproduce similar changes observed in AD by computationally reconstructing RUNX1-overexpressing iNs using Neurolucida upon immunostaining (Fig. 7, Supplementary Fig. 1-4). We used two antibody combinations (Stain 1: MAP2 (2a + 2b), Synapsin I, EGFP, DAPI; Stain 2: TUJ1, PSD95, EGFP, DAPI) to stain the neuron and synapses, and manually traced EGFP/TUJ or EGFP/MAP2 double positive cells using Neurolucida software (Fig. 2, 7). We observed clear alterations in the RUNX1-overexpressing iN's morphology regardless of the antibody combinations used (Fig. 6C, Supplementary Fig. 1-4), with RUNX1-overexpressing iNs appearing visually irregular, with a large portion of cells developing multiple protrusions from the cell body (Fig.

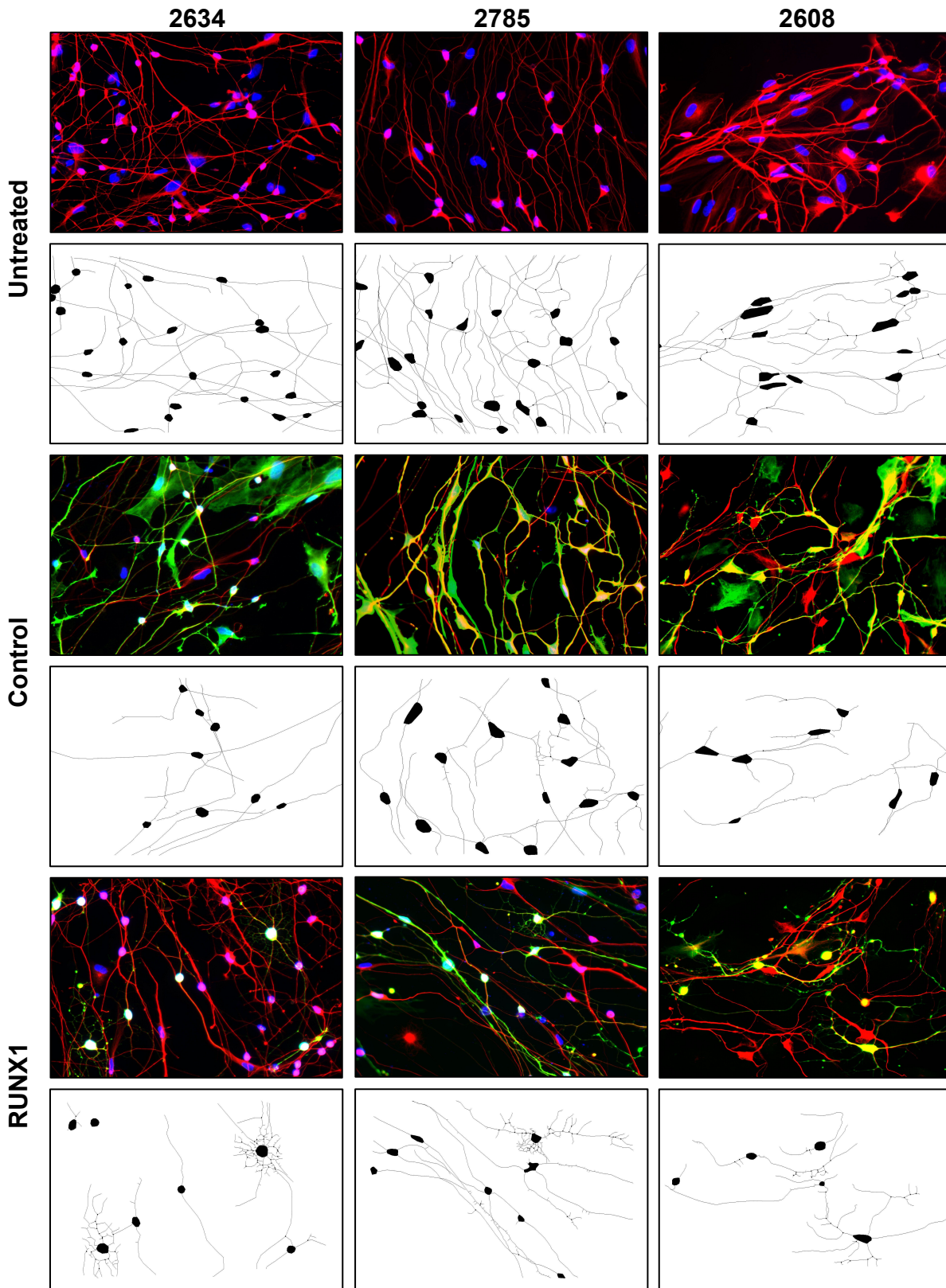


Figure 7. Morphology Reconstruction of RUNX1 Overexpressing iNs. Representative immunofluorescence image of iNs stained with DAPI, EGFP, and TUJ1 (Stain2, 20x), and their respective morphology reconstruction completed using Neurolucida software. Each column represents a donor.

6C, green arrowheads) rather than linear, bipolar dendrites seen in control and untreated iNs (Fig. 6C, Supplementary Fig. 1-4). A subset of RUNX1 iNs exhibit highly complex, thin projections surrounding the cell body akin to oligodendrocytes, while another subset appears stumped and disconnected from other cells (Fig. 6C, red and blue arrowheads respectively). Analysis of average dendritic length identified no significant differences between RUNX1-overexpressing iNs and control in either stain (Fig. 6D), but further exploration of RNA-seq data revealed that RUNX1-overexpressing iNs lose the expression of neuronal markers and intriguingly expressed the oligodendrocyte marker *OLIG1* (oligodendrocyte transcription factor 1; $\text{Log}_2\text{FC} = 2.05$, $\text{padj} = 8.31\text{E-}3$) instead (Fig. 6E) (Lu et al., 2001; Liu et al., 2021). Overall, these results demonstrate that RUNX1 robustly impairs neuronal identity by transcriptomically restricting the expression of neuron marker genes which functionally manifests as a loss of neuronal morphology.

PPAR γ Regulates Lipid Metabolism and Inflammation in neurons

PPAR γ is best known for its role as the master regulator of adipogenesis in fat cells (Aprile et al., 2014). As expected, adipogenesis emerges as the most significantly enriched hallmark when PPAR γ is overexpressed (Fig. 8B), indicating that PPAR γ retains influence on lipid metabolism in neurons. In support of that, GSEA reveals that PPAR γ is highly involved in processes related to lipid metabolism (Fig. 8A-B), as exemplified by the upregulation of gene sets including lipid droplet, fatty acid metabolism, and cholesterol homeostasis among the top ten most significantly enriched terms ($\text{padj} < 0.01$) (Fig. 8A).

The accumulation of lipid droplets is mainly associated with neurodegeneration and AD pathogenesis, but Perilipin 5 (*PLIN5*), a lipid droplet-associated protein, was found to suppress apoptosis, inflammation, and oxidative stress by activating nuclear factor erythroid 2-related factor 2 (NRF2) (Najt et al., 2020). Of the genes associated with fatty acid metabolic processes, *PLIN5* had the highest fold change ($\text{Log}_2\text{FC} = 8.68$) (Fig. 8C), suggesting a potential PPAR γ -driven protective effect. Other highly upregulated genes related to fatty acid metabolism included the drug-metabolizing enzyme *CYP4F12*

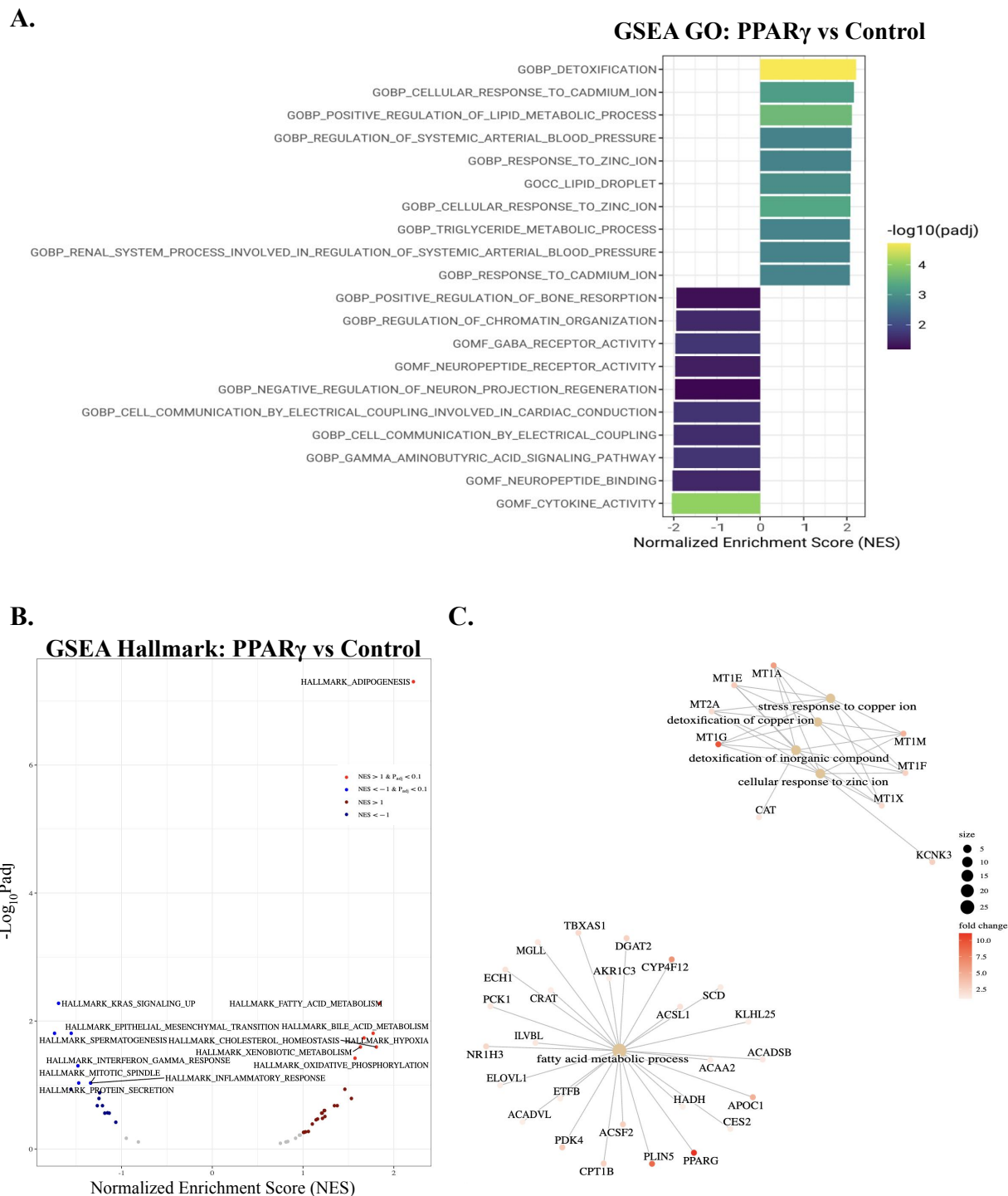


Figure 8. PPAR γ Regulates Lipid Metabolism and Inflammatory Responses in Old iNs. Gene expression profile of PPAR γ -overexpressing iNs (n=3) (A-B). GSEA of differential expression in PPAR γ -overexpressing iNs and control. (A) Bar graph showing the top ten most significantly enriched and depleted gene sets, and (B) MA plot of enriched gene sets (red, blue), with significant terms in bright red and blue. (C) GO term analysis of DGE using Gene-Concept Network (Cnetplot) depicting the linkage between genes and enriched biological concepts.

(Cytochrome P450 Family 4 Subfamily F Member 12), and member of apolipoprotein family *APOC1* (Apolipoprotein C1) ($\text{Log}_2\text{FC} = 6.34$, and 4.64 , respectively) (Fig. 8C), both of which have been associated with AD (Zhou et al., 2014; Cioffi et al., 2021).

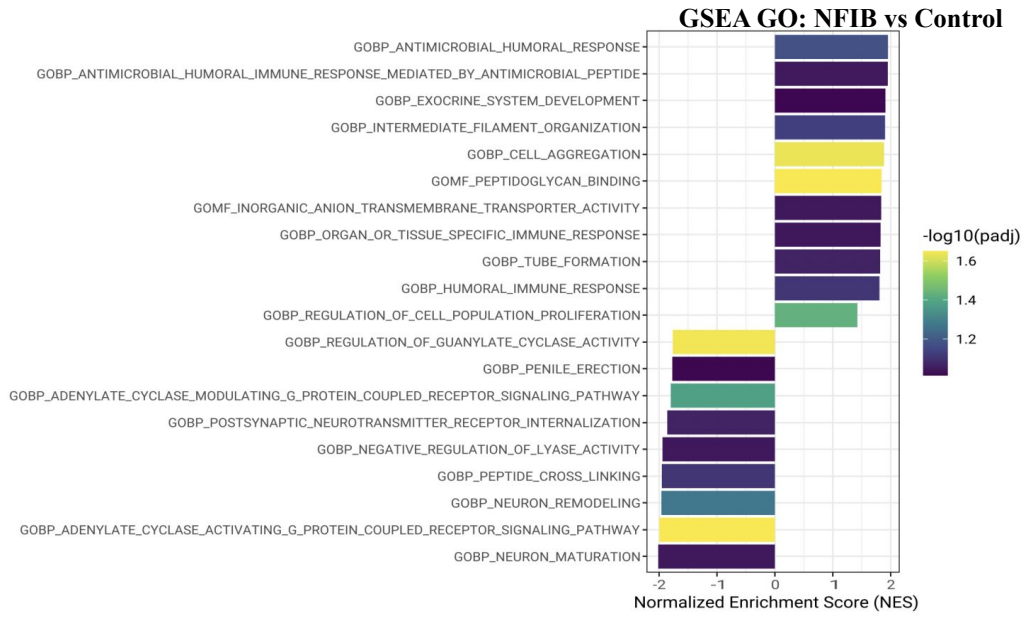
In addition to its effect on lipid metabolism, $\text{PPAR}\gamma$ also represses the expression of genes related to inflammation and interferon-gamma ($\text{IFN-}\gamma$) response (Fig. 8B), highlighting its anti-inflammatory properties. This directly opposes the phenotype seen in AD as previous transcriptomic analysis of AD neurons reported robust upregulation of genes and terms related to inflammation and inflammatory response (Mertens et al., 2021). However, it is important to take into consideration that in the AD iNs, $\text{PPAR}\gamma$ also interacts with other molecular players whose overall impact can be different. On this note, our data highlights $\text{PPAR}\gamma$'s anti-inflammatory role in neurons and suggests that $\text{PPAR}\gamma$ could have more of a protective rather than a disruptive role in AD.

NFIB Promotes the Expression of Pro-developmental Genes in Neurons

AD iN's transcriptional program resembles that of immature neurons and cells undergoing malignant transformation where genes associated with several cell cycle and cancer-related pathways, as well as stem cell and progenitor cell maintenance, are highly enriched (Mertens et al., 2021). Overexpression of NFIB in aged iNs enriches for developmental terms including regulation of cell population proliferation (Fig. 9A), and mutually upregulates many DEGs with AD iNs (Fig. 9B) (Mertens et al., 2021; Blalock et al., 2003; Annese et al., 2018). This is accompanied by the depletion of important GO terms such as neuron remodeling and maturation (Fig. 9C), and postsynaptic neurotransmitter receptor internalization (Fig. 9A), which are enriched during differentiation but depleted in AD iNs (de Leeuw et al., 2022; Mertens et al., 2021). Overexpression of NFIB also highly upregulates the expression of *HK2* ($\text{Log}_2\text{FC} = 7.97$, $\text{padj} = 1.04\text{E-}07$), a glycolytic gene commonly upregulated in AD iNs (Mertens et al., 2021), highlighting NFIB's association with the previously mentioned fate-loss and dedifferentiation in AD.

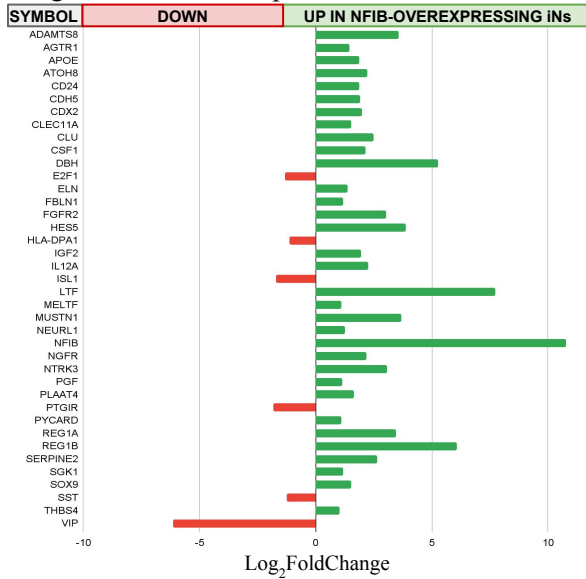
Additionally, NFIB retains its association with cancer (Denny et al., 2016) in old neurons as “KRAS_SIGNALING_DN” is the most enriched hallmark term in NFIB-overexpressing iNs (Fig. 9E). Given that the hallmark term KRAS_SIGNALING_DN comprises genes that are downregulated upon KRAS activation (Fig. 9D) (Subramanian et al., 2005; Mootha et al., 2003), the enrichment of this hallmark term indicates that KRAS signaling may be suppressed by NFIB. KRAS itself, however, is not significantly differentially expressed ($\text{Log}_2\text{FC} = 0.03$, $\text{padj} = 0.96$). From the gene list that comprises the hallmark term KRAS_SIGNALING_DN, the cancer-associated protein Keratin 15 (KRT15) is the most significantly differentially expressed in our dataset ($\text{Log}_2\text{FC} = 14.1$, $\text{padj} = 6.45\text{E-}05$), but stress-response protein serum and glucocorticoid-regulated kinase 1 (SGK1) ($\text{Log}_2\text{FC} = 1.17$, $\text{padj} = 0.02$) and the dopamine transporter solute carrier family 6 member 3 (*SLC6A3*) ($\text{Log}_2\text{FC} = 1.82$, $\text{padj} = 7.02\text{E-}12$) were the only two genes commonly upregulated (Fig. 9D) in both our data and AD iNs (Lang et al., 2018; Reith et al., 2022; Mertens et al., 2021). Overall, NFIB’s regulation of developmental and cancer-associated gene expression suggests its involvement in the adoption of transformation-associated neuronal fate instability seen in AD.

A.



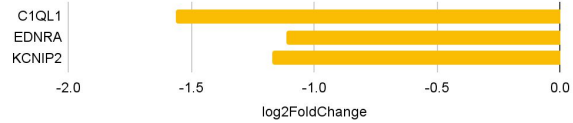
B.

Expression of Genes in the GOBP Term
Regulation of Cell Population Proliferation



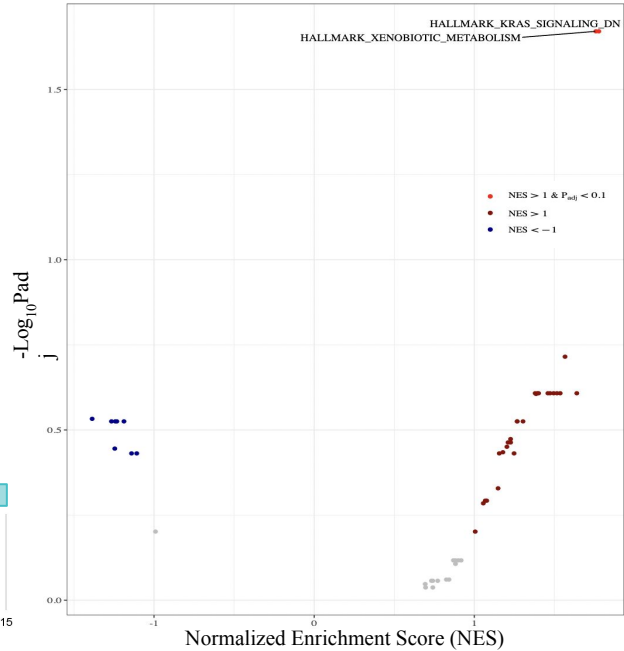
C.

Downregulation of Neuron Maturation Genes



E.

GSEA Hallmark: NFIB vs Control



D.

Genes Downregulated by KRAS Activation

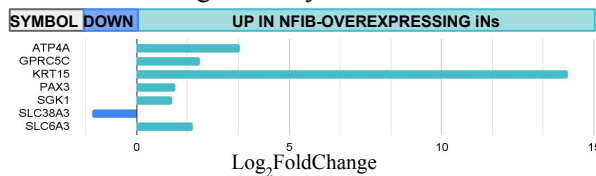


Figure 9. Characterization of NFIB-Induced Transcriptomic Changes in Neurons. GSEA analysis of DEGs in NFIB-overexpressing iNs. (A) Top ten most positively and negatively enriched GO terms. (B-D) Bar graph depicting Log₂FC of genes related to regulation of cell proliferation (B), neuron maturation (C), KRAS activation (D). (E) Volcano plot of significantly enriched hallmark terms (NES > 1 and Padj < 0.1, bright red).

DISCUSSION

YY1

Individual overexpression of YY1, RUNX1, NFIB, and PPAR γ in aged iNs caused TF-specific and distinct changes in gene expression, but YY1 had one of the strongest effects on the neuronal transcriptome overall. Our data suggest that YY1's role in aged iNs primarily revolves around the regulation of metabolic processes, a notion that concurs with its previously reported control of mitochondrial bioenergetics during development and neuronal differentiation (Zurkirchen et al., 2019). However, YY1's influence on the metabolic program becomes inverted with age as the expression of YY1 and mitochondrial genes are proportional in developing mice (Zurkirchen et al., 2019), whereas our data demonstrates a clear repression of genes associated with mitochondria-related metabolism upon YY1 overexpression in aged iNs. Given that YY1's expression decreases as neurons mature (Dong et al., 2020), it is interesting to observe increased expression of YY1 in AD iNs that undergo the metabolic changes reproducible here by the overexpression of just YY1 in old, healthy iNs (Mertens et al., 2021; Traxler et al., 2022). For example, AD patients have reported regional impairment of glucose uptake and metabolism in brain areas involved with memory and cognition, as well as obstruction of mitochondrial processes that drive oxidative phosphorylation, such as the tricarboxylic acid cycle and the electron transport chain (Mosconi, 2013; Mosconi et al., 2005; Traxler et al., 2022; De Santi et al., 2001; Xu et al., 2016).

Age-related epigenetic erosion coupled with cellular stress could induce cell cycle re-entry and dedifferentiation of neurons, presenting as loss of cell identity and adoption of a hypo-mature state as seen in AD neurons (Mertens et al., 2021). This could be due to programmed injury responses seen in many cell types where repair processes trigger cellular dedifferentiation and promote the adoption of glycolysis, which is the main metabolic program of both immature and AD neurons (Gu et al., 2020; Wagner et al., 2017; Renthal et al., 2020; Mertens et al., 2021). During injury-induced regeneration in satellite cells, YY1 facilitates the metabolic reprogramming from oxidative phosphorylation to glycolysis by repressing

the expression of mitochondrial genes and restricting oxidative phosphorylation in a similar manner to what we observe in our experiments, and activating the expression of glycolytic genes simultaneously (Chen et al., 2019). While glycolysis does not seem to be directly induced by YY1 at the transcriptomic level according to our RNA-seq data, the nuclear translocation of pPKM2, a glycolytic enzyme that regulates the Warburg-like effect in AD iNs (Traxler et al., 2022; Yang et al., 2013), coupled with the increase in lactate production, functionally demonstrates that glycolytic processes are, in fact, enhanced upon YY1 overexpression. This suggests that either the restriction of oxidative phosphorylation itself is sufficient for neurons to re-adopt the glycolytic program, or that, in addition to the repression of oxidative phosphorylation-related genes, YY1 influences post-transcriptional pathways that promote glycolysis. In the case of the aforementioned satellite cells, YY1 stabilizes the HIF1 α protein causing expression of the glycolytic gene LDHA (Chen et al., 2019), which is significantly up-regulated in AD iNs (Mertens et al., 2021) suggesting a potential role for YY1 in regulating this metabolic switch in AD iNs. However, additional experiments are required to validate this hypothesis.

It is also important to note that nuclear translocation of pPKM2 has also been associated with increased apoptotic gene expression in AD iNs, and treatment with a pPKM2-inhibitor drastically reduced the number of apoptotic AD iNs (Traxler et al., 2022). We are currently performing further experiments to investigate the effects of YY1 on apoptosis by treating YY1 overexpressing iNs with a pro-apoptotic agent ABT-737, a small molecule inhibitor of the anti-apoptotic Bcl-2 (B-cell lymphoma 2) family and quantifying cell death by performing the TUNEL (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling) assay, a directly conjugated nucleotide-fluorescent dye that marks late-stage apoptosis DNA breaks (Gorczyca et al., 1992). Adhering to this rationale, we would expect YY1-overexpressing iNs to be less apoptosis-resilient and more susceptible to cell death. A follow-up experiment would be to inhibit YY1 in AD iNs to verify whether this effect on apoptosis is reversed in diseased neurons, potentially identifying a novel target for AD.

RUNX1

RUNX1's role during the development of various cell types, including neurons, is well documented, but research on its function in differentiated, aged neurons is limited (Zagami et al., 2009). Here, we demonstrate that RUNX1 overexpression causes a loss of neuronal identity in aged iNs by downregulating a wide range of neuron-related genes and altering neuronal morphology.

In our aged iN model, overexpression of RUNX1 inhibited axonal growth, a process facilitated by RUNX1 during development (Marmigère et al., 2006), and downregulated various neuronal markers such as microtubule-associated protein 2 (*MAP2*) and neuronal differentiation (*NeuroD*), which are also downregulated in AD (Mertens et al., 2021). Researchers in our lab previously reported a return-to-immaturity in AD iNs and discussed how a hypo-mature state in neurons could lead to impaired cellular resilience and a dismantling of apoptotic brakes present in mature neurons that prioritize maintenance and longevity (Mertens et al., 2021, Traxler et al., 2021). The downregulation of genes related to nervous system development and loss of neuronal markers caused by RUNX1 overexpression could contribute to this dedifferentiation-linked destabilization of the neurons seen in AD, and increase their susceptibility to cell death (Traxler et al., 2021).

Furthermore, the loss of neuronal identity in RUNX1-overexpressing cells functionally translated to an altered neuronal morphology where a large portion of the cells developed additional protrusions from the cell body and others resemble a different cell type altogether. Given that a neuron's principal operation is to communicate via electrochemical signals between one another, neuronal morphology is an important dictator of network connectivity and predictor of neuronal health and function (Peng et al., 2021; Udvary et al., 2022). Alterations in neuronal morphology are common among neurological diseases, and in AD, neurons undergo spine loss and shortening of the dendrites which correlates to loss of synaptic plasticity and neurodegeneration (Goikolea-Vives and Stolp, 2021; Tsai et al., 2004; Ji et al., 2003; Pelucchi et al., 2020). Although overexpression of RUNX1 alone did not fully recapitulate the morphological changes seen in AD, the loss of healthy neurotypical morphology could be indicative of

altered neuronal function, a notion supported by the downregulation of genes related to synaptic transmission from our RNA-seq. However, further experiments on neuronal activity are required.

Additionally, we observed, although to a very low extent, an oligodendrocyte-like morphology in some RUNX1-overexpressing iNs cultures. It is possible that the adoption of the oligodendrocyte fate specifically is not a direct effect of RUNX1, but a limitation of our model. To improve the conversion efficiency of fibroblasts to neurons, our protocol utilizes the UNA construct, a doxycycline-inducible tetOn system cassette that drives the expression of the pioneer transcription factors neurogenin2 (Ngn2) and achaete-scute homolog 1 (Ascl1), that can induce opening of the chromatin around promoters where the small molecules in the neural conversion media bind to promote conversion from fibroblast to neuron (Herdy et al., 2019; Velasco et al., 2017; Chanda et al., 2014; Păun et al., 2023). When considered with our RNA-seq results, one hypothesis is that the RUNX1 inhibition of pro-neuronal gene expression could limit or reverse the complete adoption of the neural fate and, with the chromatin at a more accessible state, forces the cells to adopt an alternate fate. In this case, the cells developed a similar morphology to oligodendrocytes and upregulated the expression of oligodendrocyte markers instead (Fig. 6E). This raises the question of how the transcriptional changes caused by RUNX1 overexpression here would manifest in naturally occurring, mature neurons, which, according to research in adult mouse brains, resulted in slight reduction of dendritic morphology and complexity, consistent with our findings (Fukui et al., 2018). Therefore, while the adoption of another cell fate could partially obscure RUNX1's true effects on neuronal morphology and render further quantification and analysis difficult, our overall data concur with the notion that RUNX1 can contribute to the dedifferentiation of neurons in AD by heavily impeding the expression of various pro-neuronal genes.

PPAR γ

In concurrence with PPAR γ 's well known role as the master regulator of adipogenesis in fat cells (Aprile et al., 2014), our data show that PPAR γ highly influences the expression of genes related to various lipid homeostatic processes in aged neurons. Alterations in lipid metabolism and fatty acid

composition have been associated with both healthy aging and AD (Yu et al., 2020). In the aging brain, the lipidome naturally becomes increasingly complex, and the majority of cortical lipid concentrations change in an age-dependent manner (Yu et al., 2020). However, the AD brain's metabolite profile is unique and distinct from that of age-matched healthy controls (Snowden et al., 2017, Traxler et al., 2022). Despite the differences, changes in lipid metabolism appear to be associated with AD pathogenesis as metabolite levels in asymptomatic AD patients (those categorized by the researchers as patients with AD neuropathy but no cognitive impairment) are intermediary between AD and healthy patients, and changes in fatty acid composition in the brain preceded behavioral deficits in AD mouse models (Snowden et al., 2017, Bai et al., 2020). Given that our data show that PPAR γ transcriptomically regulates lipid metabolism in aged neurons, an environment where lipid concentrations are already altered, it is possible that the downstream effects caused by PPAR γ -induced changes in gene expression could either tip the already-vulnerable cells over the pathological threshold towards AD or help prevent them from doing so.

A similar framework extends towards alterations in cholesterol homeostasis, another hallmark term upregulated in our data, where uncontrolled production of cortical cholesterol reportedly exacerbated the AD phenotype by increasing neuroinflammation and accelerating oxidative damage and cognitive decline (Yin, 2023). Concurrently, high cholesterol levels during mid-life increased the risk of developing AD in old age, and reduction of neuronal cholesterol significantly reduced beta-secretase activity and production of insoluble A β (Kivipelto et al., 2002; Schneider et al., 2006), further highlighting the importance of maintaining balanced lipid levels in the brain and emphasizing the potential harm of PPAR γ -induced alterations in lipid metabolism. However, the GSEA annotated gene set names do not specify if the enriched lipid metabolism-related terms refer to catabolic or anabolic pathways. For example, the official description for ‘GOBP_POSITIVE_REGULATION_OF_LIPID_METABOLIC_PROCESS’ given by AmiGO is “any process that activates or increases the frequency, rate or extent of the chemical reactions and pathways involving lipids”, which could encompass both catabolic and anabolic processes (Ashburner et al., 2000; Gene Ontology Consortium, 2021). Therefore, in order to more confidently infer PPAR γ 's contribution to

AD, experiments and analysis of transcriptomic data are required. Additional experiments could include:

1. Further analysis of our RNA-seq with Compass, an algorithm developed for the characterization of a cell's metabolic state based on RNA-seq, to predict which metabolic pathways specifically are up or downregulated;
2. Metabolic profiling of iNs overexpressing PPAR γ to identify which metabolites increase or decrease in level.

In addition to its influence on lipid metabolism, PPAR γ also represses genes related to inflammation and inflammatory response. While this is in direct opposition to the highly inflammatory phenotype observed in AD, the anti-inflammatory effects of PPAR γ are well documented in various cell types, highlighting PPAR γ 's neuroprotective functions (Mertens et al., 2021; Glass and Saijo, 2010; Odegaard et al., 2007; Straus and Glass, 2007). Notably, PPAR γ is a downstream effector of some NSAIDs and can promote A β clearance by regulating microglial activation (Lehmann et al., 1997; reviewed by Shie et al., 2009), and PPAR γ agonists such as rosiglitazone have been proposed as a potential clinical treatment against A β accumulation (in t' Veld et al., 2001, Jiang et al., 2008). PPAR γ agonists have also been reported to prevent aberrant neuronal cell cycle re-entry induced by microglia-mediated inflammation, although unable to reverse pre-existing hypo-maturity (Mertens et al., 2021; D'Angelo et al., 2017; Bonda et al., 2010; Varvel et al., 2009). While evidence supporting the efficacy of PPAR γ agonists as treatment remains conflicting, our data is in support of PPAR γ 's neuroprotective and anti-inflammatory effects.

NFIB

NFIB is well known for its association with various types of cancer, where it paradoxically suppresses or promotes cancer metastasis depending on cancer type (Denny et al., 2016; Fane et al., 2017; Zhou et al., 2014; Becker-Santos et al., 2017). Our data demonstrate that NFIB preserves its cancer-associated roles in old iNs as overexpression of NFIB promotes the expression of genes inhibited by KRAS activation, potentially suppressing KRAS signaling, while also strongly upregulating the

expression of KRT15 ($\text{Log}_2\text{FC} = 14.1$, $\text{padj} = 6.45\text{E-}05$), a gene associated with poor cancer prognosis (Rao et al., 2020; Lin et al., 2020).

AD and cancer share many parallels despite seeming like disparate diseases, and cancer-related biological processes such as the Warburg effect are observed in AD (Traxler et al., 2022; Mertens et al., 2021). One commonality is that cells from both diseases develop cellular plasticity where they lose their specialized identities and adopt a less differentiated phenotype akin to stem cells, rendering cells vulnerable to pathological development (Mertens et al., 2021; Poplawski et al., 2020; Li et al., 2019). Although there is a clear lack of research directly associating NFIB with KRAS or KRT15, as oncogenes, KRAS and KRT15 are well known regulators of cell proliferation and play crucial roles during malignant transformation (Ievlev et al., 2023; Huang et al., 2021). When considering NFIB's robust influence on these oncogenes in conjunction with the downregulation of neuronal maturity genes and enrichment of developmental terms, our data suggests NFIB's involvement in the aforementioned dedifferentiation of neurons and gain in cellular plasticity characteristic of AD.

Among others, overexpression of NFIB downregulates *KCNIP2*, a gene associated with neuron differentiation, and robustly upregulates the stem cell-associated glycolytic gene *HK2* ($\text{Log}_2\text{FC} = 7.97$), which together with the associated transcriptional changes are indicative of a loss of identity and potential facilitation of the Warburg effect. Similarly, the downregulation of genes within the GO term postsynaptic neurotransmitter receptor internalization could infer a loss of neuronal function, although further experiments such as examining NFIB-induced changes in electrophysiology are required.

Additionally, a highly accessible chromatin landscape is characteristic of cancer cells and immature stem cells that are able to differentiate into various cell types compared to the more closed, inaccessible chromatin seen in differentiated cells (Mertens et al., 2021; Wu and Sun, 2006; Lu et al., 2020). Mertens and colleagues (2021) found that AD iNs possess a similar hypo-mature chromatin landscape, with an opening of chromatin around promoters of genes related to signaling and nervous system development. Considering that NFIB is a pioneer factor and can cause a global increase in chromatin accessibility in lung cancer (Denny et al., 2016), we also performed ATAC-seq to probe

whether a similar effect can be reproduced in neurons. Although this data is still currently being analyzed and further verified, at this preliminary stage, we observe a widespread opening of the chromatin in NFIB-overexpressing iNs. Therefore, taken together with our RNA-seq data, NFIB could contribute to the loss of mature resilience by inducing a widespread increase in DNA accessibility while simultaneously regulating the transcription of developmental and neuron maturation genes. When considered in the uncontrolled, complex environment found in AD neurons where a multitude of factors are working in tandem, the opening of the chromatin caused by NFIB could prime the DNA and allow other transcription factors to bind to previously inaccessible areas and express genes phenotypic of AD.

CONCLUSION

Here, we investigated the contribution of YY1, RUNX1, NFIB, and PPAR γ to AD pathology in aged iNs and demonstrated that the four TFs influence various biological aspects that promote the adoption of the hypo-mature cellular program (Figure 10). YY1 facilitated the Warburg-like metabolic switch from oxidative phosphorylation to glycolysis, RUNX1 and NFIB restricted neuronal identity by downregulating expression of mature neuron-related genes, and PPAR γ both downregulated inflammatory response genes and mediated lipid metabolism. Although these roles have been previously observed in various cell types, the high context specificity of these TFs hindered the ability to confidently determine their function in aged neurons prior to this study. By utilizing donor-derived iNs as a cellular model that captures biological changes associated with aging, this thesis has provided deeper human-applicable insight into how, in conjunction with age-related changes such as epigenetic erosion, these putative TFs could facilitate the transformation-associated ‘return-to-immaturity’ that could provoke the pathological hallmarks of AD.

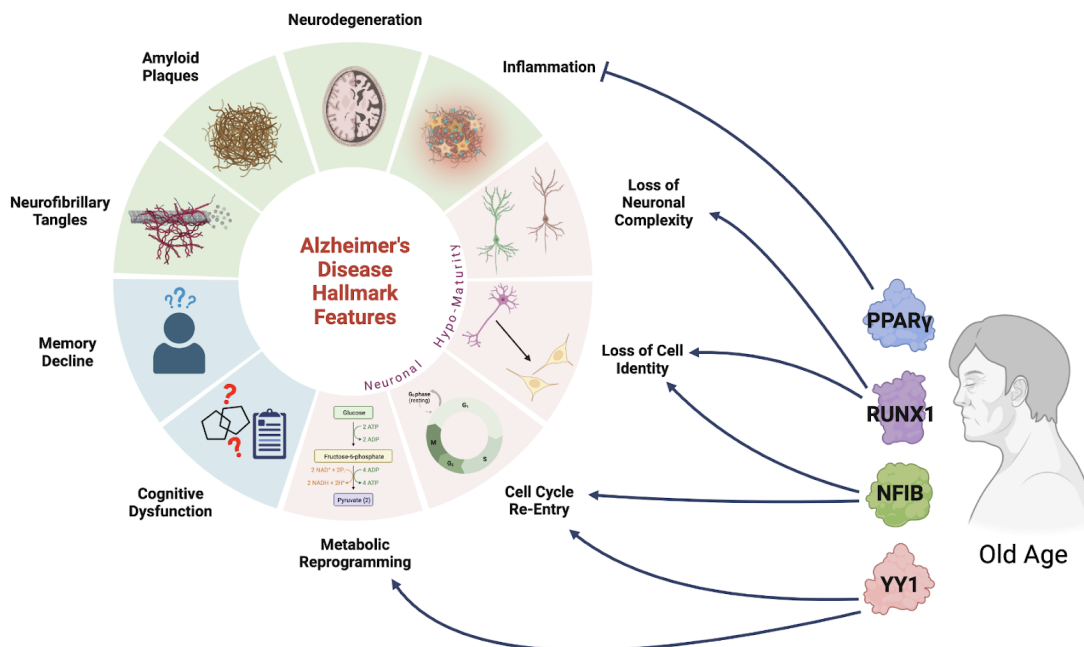
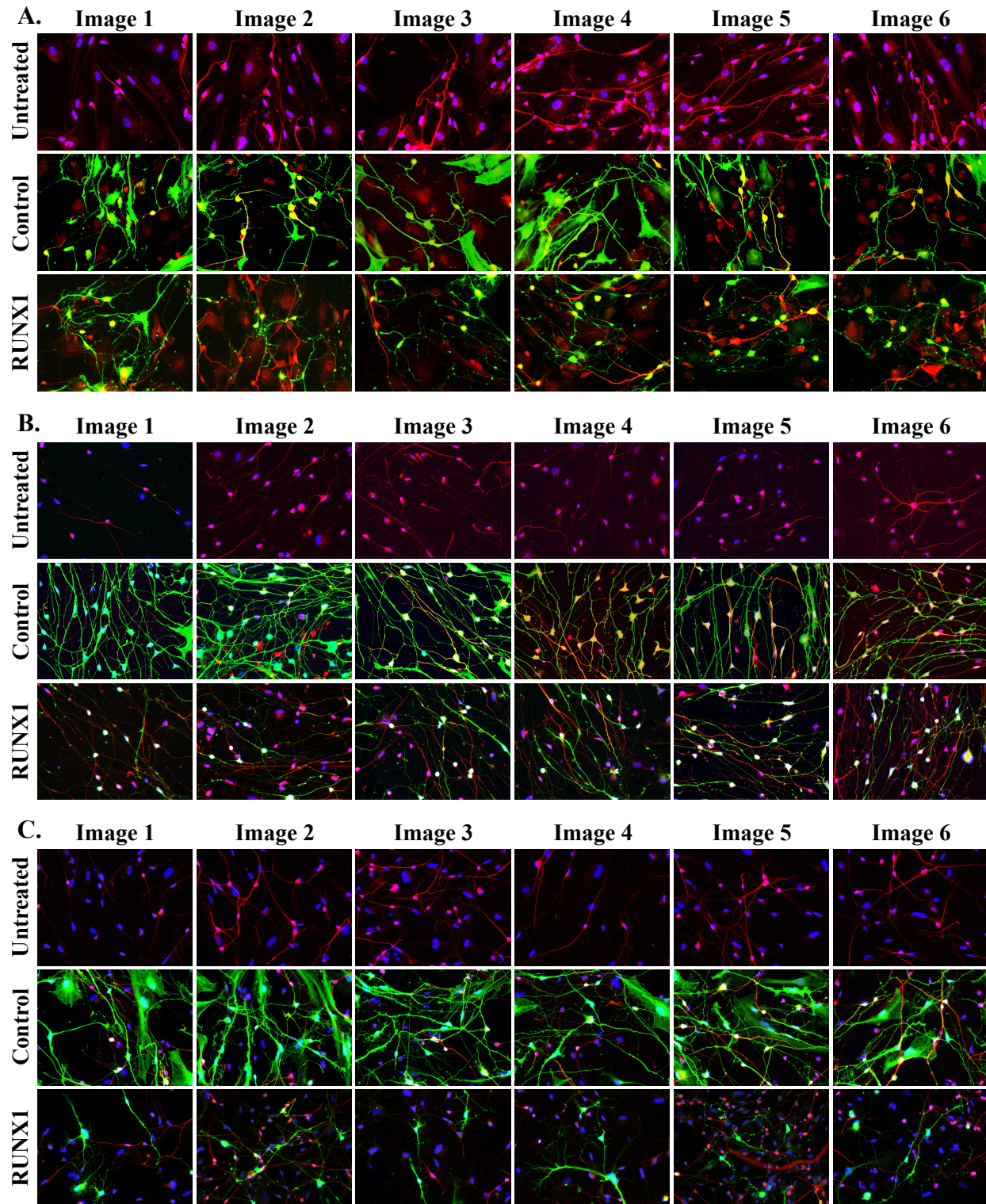
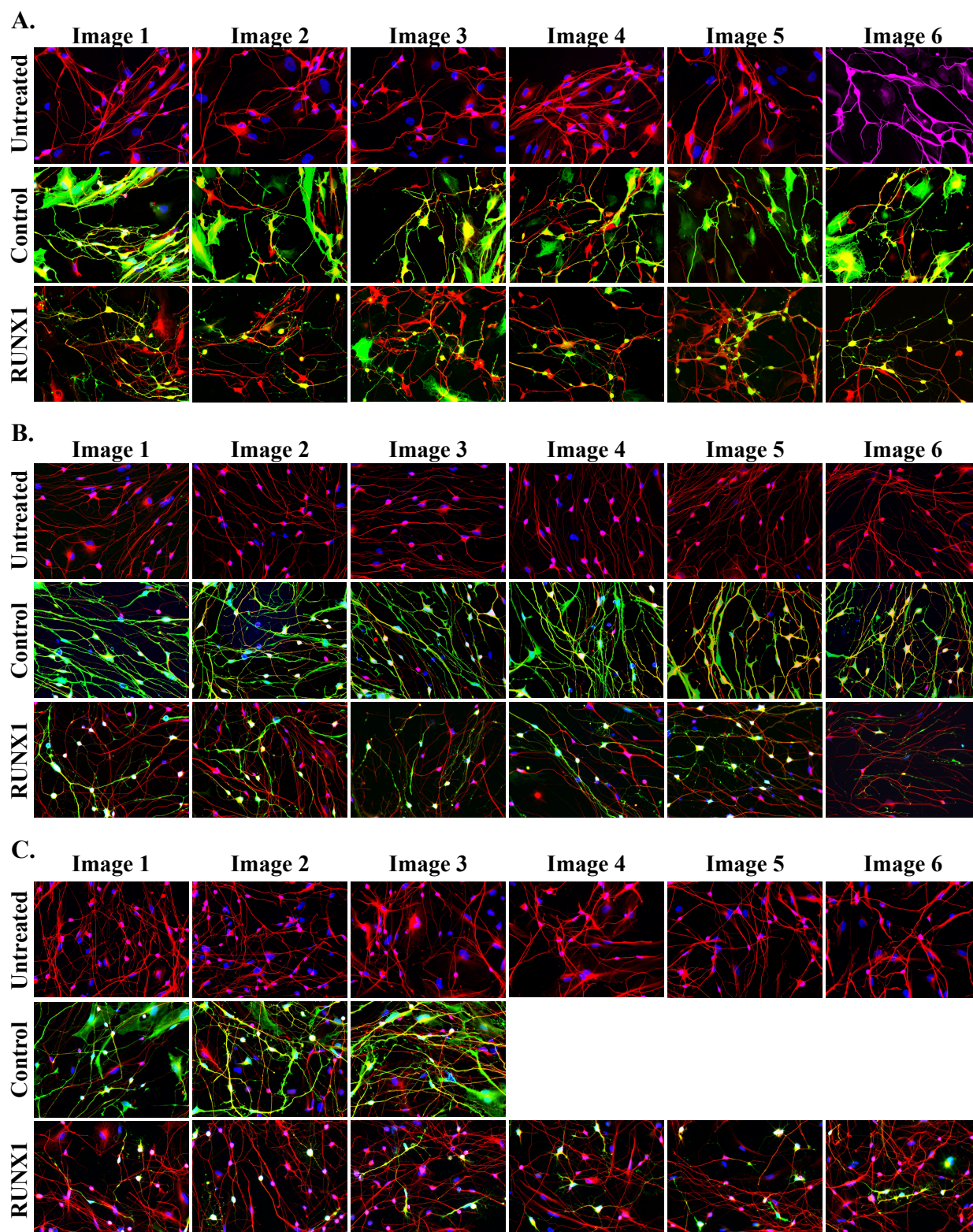


Figure 10. YY1, RUNX1, NFIB, and PPAR γ Facilitates Neuronal Hypo-Maturity. Summary of findings showing the contribution of each transcription factor to AD and the hypo-mature state, created with BioRender. Components of the wheel identify important pathological hallmark features of AD; green sector = histological hallmarks of AD, blue sector = fundamental symptoms of AD, and pink sector = phenotypic manifestation of AD-related hypo-mature cellular program.

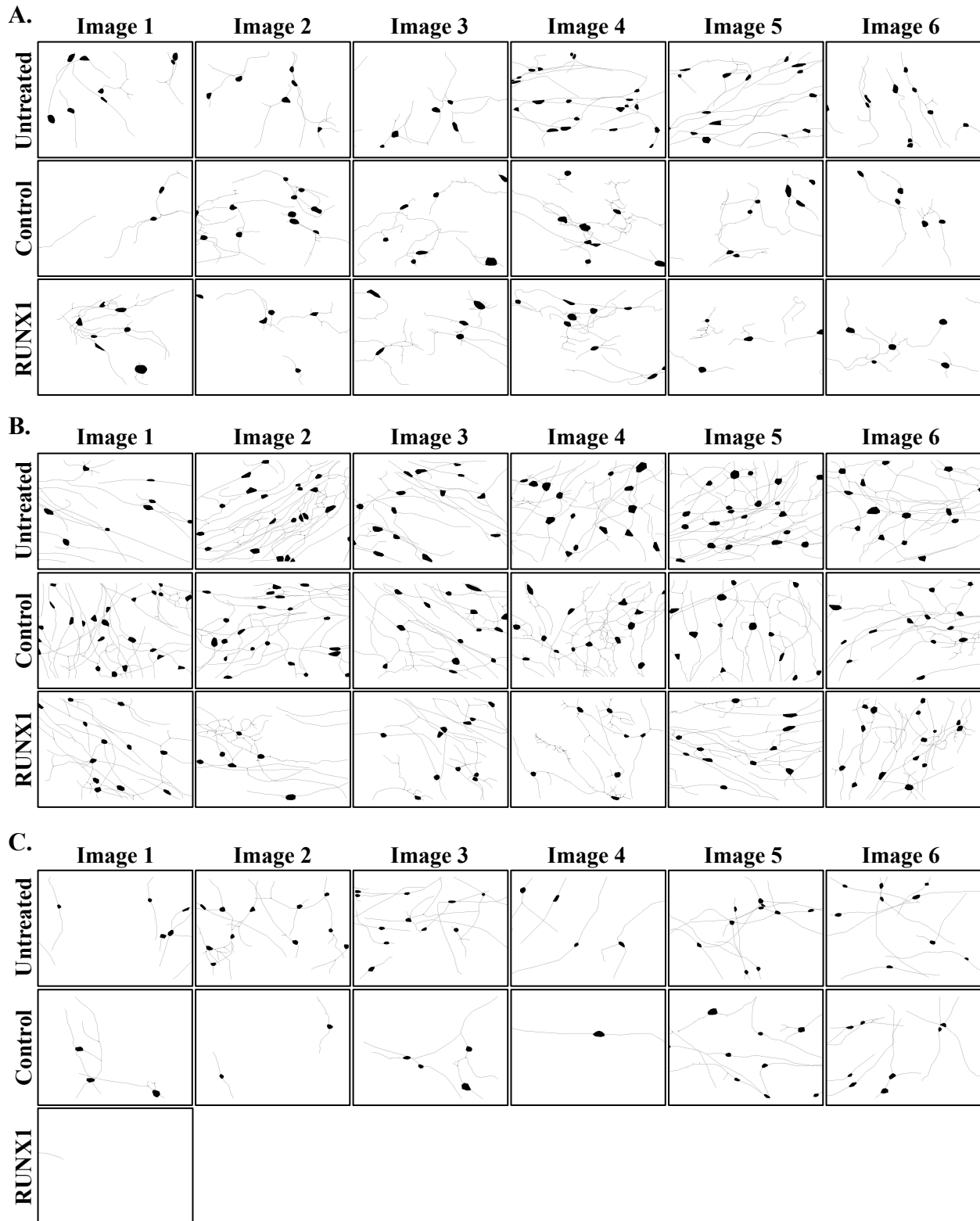
SUPPLEMENTARY FIGURES



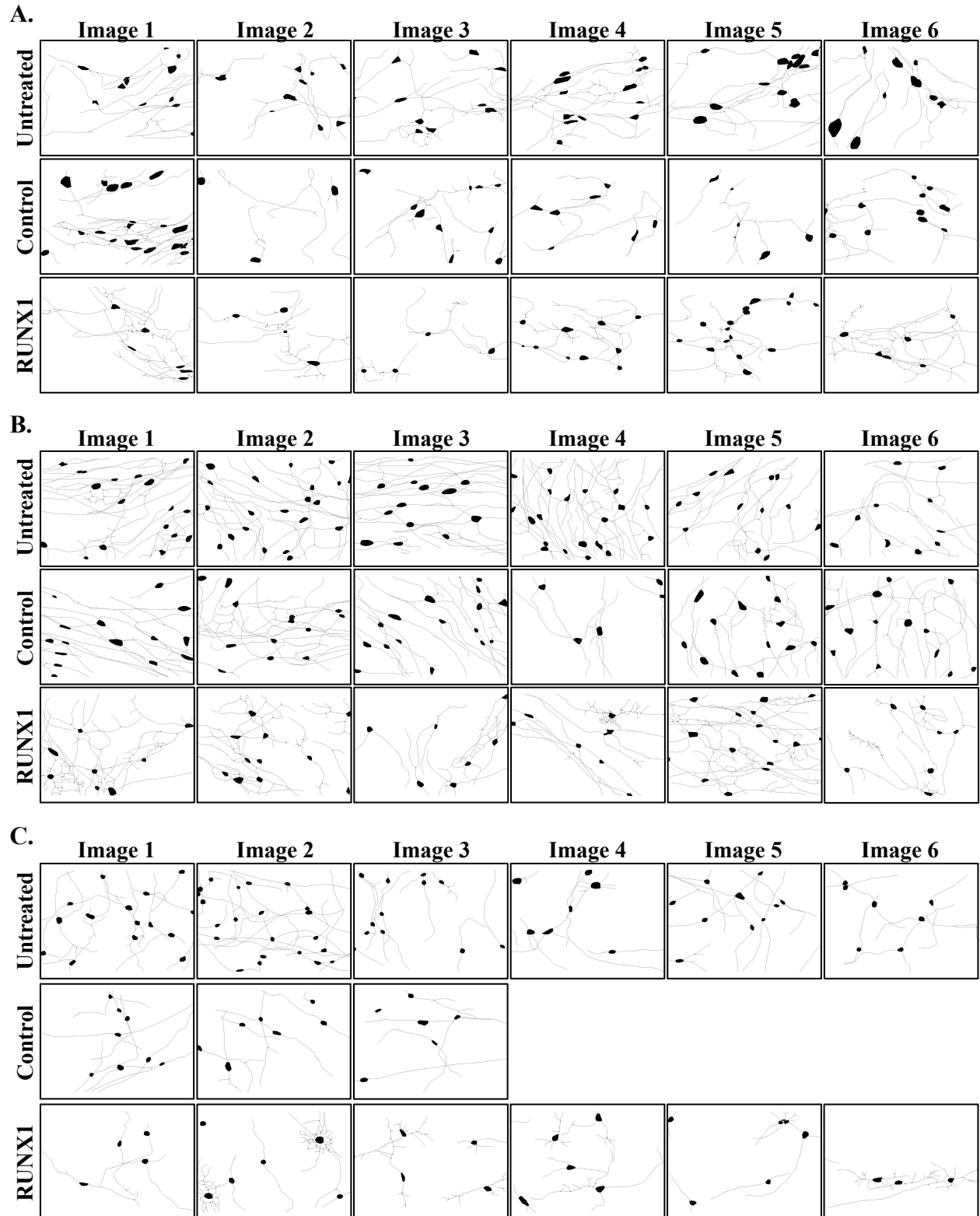
Supplementary Figure 1. **Composite Fluorescent Images for Morphological Reconstruction of RUNX1-Overexpressing iNs Stained With Stain Combination 1.** Neuronal morphology reconstructed using the Neurolucida software based on DAPI, EGFP, MAP2a+2b, and Synapsin 1 (Stain 1). Each column represents a technical repeat. Donors **(A)** 2608, **(B)** 2785, **(C)** 2634.



Supplementary Figure 2. **Composite Fluorescent Images for Morphological Reconstruction of RUNX1-Overexpressing iNs Stained With Stain Combination 2.** Neuronal morphology reconstructed using the Neurolucida software based on DAPI, EGFP, TUJ and PSD-95 (Stain 2). Each column represents a technical repeat, images 1-3 and 4-6 from two different wells. Donors **(A)** 2608, **(B)** 2785, **(C)** 2634. Missing images were due to contamination in the well (C).



Supplementary Figure 3. **Morphology Reconstruction of RUNX1-Overexpressing iNs Stained With Stain Combination 1.** Neuronal morphology reconstructed using the Neurolucida software based on DAPI, EGFP, MAP2a+2b, and Synapsin 1 (Stain 1). Each column represents a technical repeat. Donors (A) 2608, (B) 2785, (C) 2634. Missing images are due to lack of EGFP/MAP2a+2b double positive cells.



Supplementary Figure 4. **Morphology Reconstruction of RUNX1-Overexpressing iNs Stained With Stain Combination 2.** Neuronal morphology reconstructed using the Neurolucida software based on DAPI, EGFP, TUJ and PSD-95 (Stain 2). Each column represents a technical repeat. Donors (A) 2608, (B) 2785, (C) 2634.

All of the work included in this thesis is under consideration for a publication currently being prepared for submission. The thesis author is a researcher working under Dr. Raffaella Lucciola and Dr. Fred H. Gage, and co-author of this material.

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