STUDY OF THE ROLE AND REGULATION OF MASTER TRANSCRIPTION FACTOR, REST, REVEALS LIMITATIONS OF AN *IN VITRO* HUMAN AGING SYSTEM

By

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List of Abbreviations

REST	RE1-Silencing Transcription Factor
NRSF	Neuron Restrictive Silencer Factor
AD	Alzheimer's Disease
cDNA	Complementary DNA
iNs	Induced neurons
HEK-293	Human embryonic kidney 293 cells
GLRA1	Glycine receptor alpha 1
Rbfox3	RNA binding protein fox 1 homolog 3
shRNA	Small hairpin RNA
SNAP25	Synaptosomal-associated protein, 25 kDa
NeuN	Neuronal Nuclei
UTR	Untranslated region
iPSCs	Induced pluripotent stem cells
RE1	Repressor Element 1
NRSE	Neuron Restrictive Silencer Element
NPCs	Neural progenitor cells
BMP2	Bone morphogenetic protein 2
NgCAM	Neuronal cell adhesion molecule
SCG10 or STMN2	Stathmin 2
BDNF	Brain derived neurotrophic factor
Nav1.2 or SCN2A	Type II voltage gated sodium channel
NPAS4	Neuronal pas domain containing protein-4
PC12	Rat pheochromocytoma cells
mESCs	Mouse embryonic stem cells

miR	MicroRNA
H4K20Ac	Histone H4 acetylation at lysine 20
CHX	Cycloheximide
β-TRCP	Beta-transducin repeats-containing proteins
UPS	Ubiquitin Proteasome System
XBR	X2 box repressor
EZH2	Enhancer of zeste homolog 2
CAT	Chloramphenicol acetyltransferase
ORF	Open reading frame
HDACs	Histone deacetylases
SWI/SNF	SWItch/Sucrose Non-Fermentable
HAUSP or USP7	Herpesvirus-associated ubiquitin-specific protease
SCF	Skp, Cullin, F-box containing complex
KDM1A or LSD1	Lysine (K)-specific demethylase 1A
SIN3A	Sin3 Transcription Regulator Family Member A
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation followed by next generation sequencing
qChIP	Quantitative chromatin immunoprecipitation
SRRM4	Serine/arginine Repetitive Matrix 4
ASOs	Antisense oligonucleotides
HD	Huntington's disease
PTMs	Post translational modifications
NeuroD	Neurogenic differentiation factor
SACO	Serial Analysis of Chromatin Occupancy

ZFP36L2	Zinc finger protein 36-like 2
MAD2	Mitotic arrest deficient 2
CTDSP1	Carboxy terminal domain RNA polymerase II peptide A small phosphatase 1
ERK 1/2	Extracellular signal regulated kinase 1/2
PRC2	Polycomb repressive complex 2
H3K27Me3	Tri-methylation of lysine 27 on histone H3
KDM1A	Lysine demethylase 1A

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Abstract of the dissertation

Nervous system development is a highly complex process involving multiple cellular transitions from that of an embryonic pluripotent state to that of a terminally differentiated neuronal or non-neuronal state. The precise control of gene expression by transcription factors is critical for facilitating the systematic acquisition and maintenance of the appropriate cellular fates. One such essential transcription factor is the RE1-Silencing transcription factor (REST). Discovered as a transcriptional repressor of neuronal genes in non-neuronal cells, REST regulates thousands of genes required for the terminally differentiated neuronal phenotype, and is a key regulator of mammalian neurogenesis. REST is highly expressed in embryonic stem cells, and is rapidly downregulated during their *in vitro* differentiation into neurons, where removal of REST from chromatin ensures terminal neuronal differentiation. While most REST studies have been conducted in the context of mammalian embryonic development, recent studies have suggested novel roles for REST in the mature post-natal brain, including the aging human brain. Moreover, REST has been proposed to be neuroprotective during human brain aging, a compelling hypothesis that has not been independently tested. Due to the limitations in obtaining resected fresh human brain tissue for aging research, many research groups have invested in developing alternate *in vitro* models which involves the conversion of accessible human primary cells, such as dermal fibroblasts, into neurons, using different cellular reprogramming approaches. Here, in my dissertation research, in order to test hypotheses regarding the role of REST in human brain aging, I utilized one particular *in vitro* model of aging human neurons, also known as induced neurons (iNs) differentiated from dermal fibroblasts using microRNAs. Prior studies have identified new mechanisms related to REST regulation during the microRNAs-mediated reprogramming of human dermal fibroblasts to iNs. Moreover, human iNs derived from individuals of

different ages have previously been reported to preserve age-associated molecular signatures, including at the level of the epigenome. Collectively, these findings implicated the use of human iNs as a model to study REST function in aging. However, unexpectedly, using a variety of molecular and biochemical analyses, I was not able to reproduce fundamental published findings related to REST in this aging model, precluding my ability to gain insights into REST function using this system. In particular, post translational modifications assigned to REST, and the extent to which human iNs reached neuronal maturity, were not replicated. None-the-less, my work does highlight the limited extent to which iNs truly reflect neuronal maturity of the human brain, using REST function as a ruler. The findings from my work may prove useful to investigators studying other transcription factors in human iNs.

Chapter 1: Introduction

The generation of neurons during nervous system development is dependent on the precise spatial and temporal control of gene expression by transcription factors. During this process, there is an orchestrated differentiation of cells from an embryonic, pluripotent state to neural progenitors and finally to that of terminally differentiated neurons. In order for these stages to progress, extrinsic neurogenic cues and intrinsic cellular pathways must work in a coordinated manner, processes often mediated by transcription factor cascades. For example, during embryogenesis, signaling by the Notch pathway induces expression of the Hes genes in neural progenitors, which repress pro-neural transcription factors, such as Mash1, thereby maintaining their proliferative state (Kageyama & Ohtsuka, 1999). Conversely, bone morphogenetic protein 2 (BMP2) signaling induces expression of Mash1 in neural progenitors resulting in cell-cycle arrest and activation of neuronal gene programs (Bertrand, Castro, & Guillemot, 2002). Similar studies on other pro-neural factors such as the NeuroD genes, helped understand neuronal specification mechanisms (Lee et al., 1995). It was, however, the discovery of the RE1 silencing transcription factor (REST) that provided a more complete understanding of all the sequential steps underlying neurogenesis.

The discovery of REST drew attention, for the first time, to the role of an anti-neural factor in regulating mammalian neurogenesis (Ballas et al., 2005). First identified as a transcriptional repressor of the type II voltage-gated sodium channel in non-neuronal cells (Chong et al., 1995; Schoenherr & Anderson, 1995), REST was later shown to act as a master regulator of hundreds of neuronal genes essential to nervous system function, such as voltage-gated ion channels, neurotransmitter release, post-synaptic signaling, neurotrophins, axon guidance, etc., and required for the terminal differentiated phenotype of post-mitotic neurons (Bruce et al., 2004; Mortazavi et al., 2006; Johnson et al., 2007;

Otto et al., 2007). REST is highly expressed in mouse embryonic stem cells (mESCs), where it represses the large network of neuronal genes, and is rapidly downregulated during its *in vitro* differentiation into neural progenitors, detected only by chromatin immunoprecipitation of its binding to target genes. Removal of REST from chromatin in neural progenitors, close to the time they exit the cell cycle, relieves its repression of neuronal genes and terminal neuronal differentiation ensues (Ballas et al., 2001). This downregulation of REST is mediated by both post-transcriptional and post-translational mechanisms. The post-transcriptional mechanism has not been thoroughly investigated, but the post-translational mechanism involves the activity of a specific E3 ubiquitin ligase known as β-TRCP, that degrades it through the proteasome (Westbrook et al., 2008; Huang et al., 2011; Nesti et al.,2014). Moreover, owing to the unusually large length of the REST binding site, Repressor Element 1, (RE1; 21 bp) compared to other transcription factor motifs, REST has been the subject of bioinformatics approaches for genome-wide transcription factor binding studies (Bruce et al., 2004; Mortazavi et al., 2006) including the first genome wide chromatin binding assays, Serial Analysis of Chromatin Binding (SACO) (Otto et al., 2007), and Chromatin Immunoprecipitation followed by next generation sequencing (ChIP-Seq) (Johnson et al., 2007).

Since its initial discovery, REST has been studied extensively in the formation of the embryonic nervous system using mouse models. Recently, however, new roles for REST were discovered in the post-natal mouse brain (Sun et al., 2005; Kuwabara et al., 2005; Gao et al., 2011; Nechiporuk et al., 2016) and proposed in the mature adult human brain (Lu et al., 2014; McGann et al., 2021). A recent surprising discovery from other members of our lab was that REST regulation was distinct in the adult mouse and human brain (McGann et al., 2021). Specifically, their study utilized archived human brain tissue samples to show that REST levels rise during aging in the human hippocampus, in complete contrast to that of the mouse hippocampus where REST decreases with age.

Further, the REST target genes in the aged human hippocampus were non-canonical and distinct from its target genes in the mouse hippocampus. One caveat of this study, however, is that because of the drop in REST levels in aging mice, comparisons were necessarily between aged human brain and juvenile mice (5-weeks of age). Because REST has been implicated in mechanisms underlying human longevity (Lu et al., 2014; Zullo et al., 2019) and neurodegenerative diseases, such as Alzheimer's (Lu et al., 2014; Mertens et al., 2015; Meyer et al., 2019), a more complete understanding of the mechanistic roles for REST in healthy human neuronal aging, merits more study. Since live human brain neurons cannot be accessed for this purpose, I became interested in using alternate *in vitro* systems that have been developed to model the aging human brain using cellular reprogramming approaches (Takahashi, 2014; Wang et al., 2021). In researching them, I found a recently developed model of human aging neurons, derived by direct cellular reprogramming of human dermal fibroblasts using microRNAs, to be most suited to study REST in aging. Our rationale for choosing this model is based on the preservation of an age-associated epigenomic feature, also known as the epigenetic clock (Horvath, 2013), in the reprogrammed human neurons (Huh et al., 2016). A more detailed description of the epigenetic clock analysis in aging and its maintenance in the microRNAs-derived model of human neurons is described in the introduction to Chapter 3.

Finally, while recent studies are focusing on the novel human-specific repressor roles for REST, much less is known about the mechanisms that regulate its levels in human cells. Recently, REST levels were proposed to be regulated by a novel post-translational mechanism, involving the methylation of REST at lysine (K) 494 by a methyltransferase enzyme, known as enhancer of zeste homolog 2 (EZH2) (Lee et al., 2018). However, whether methylation of REST worked in concert with β-TRCP, the E3 ubiquitin ligase recruited by REST for its degradation, remains unknown. Since the correct

maintenance of REST levels is crucial for its function as a repressor, especially during brain development, a deeper understanding of its post-translational regulatory mechanisms is needed.

Here, in my dissertation I studied two different aspects of the human REST protein, the first related to its role in brain aging, and second, the post-translational mechanisms that help regulate its levels in human cells. In my first study, to gain mechanistic insights into REST in human brain aging, I utilized an *in vitro* model of human neurons, also known as induced neurons (iNs) derived from primary human dermal cells using a microRNAs-based cellular reprogramming approach (Yoo et al., 2011; Richner et al.,2015). Using molecular and biochemical techniques, I characterized REST levels and its chromatin binding in aged human iNs, only to unexpectedly identify major limitations associated with REST, making them unsuitable to study mechanisms of REST. My second study focused on examining the role of the recently proposed amino-acid residue (K494) in REST protein targeted for methylation by EZH2 (Lee et al., 2018), in affecting its stability in human cells. To do so, I utilized a mutated version of human REST protein at K494 (K494>A), and tested whether it affected REST stability, using *in vitro* protein degradation assays. My findings contradicted the previous work, wherein the K494 site in human REST protein did not affect its stability. I also identified that mutation of the K494 site (K494>A) in REST did not affect its recruitment of the E3 ubiquitin ligase, β-TRCP, for degradation in human cells.

During the remaining of this chapter, I will provide a detailed background on REST, starting with its discovery (1.1), a description of its functional domains and interactions, fundamental to understanding how it facilitates neuronal gene repression (1.2), an overview of its role as a key regulator of mammalian neurogenesis (1.3), followed by a detailed description of the molecular mechanisms known to regulate REST expression and their significance in the formation and maintenance of neurons (1.4), and lastly a

description of the recent discoveries related to REST function in the adult brain with a focus on the proposed roles for REST in the aging human brain and Alzheimer's disease (1.5). In Chapter 2, I describe the materials and methods used in my dissertation. In Chapter 3, I describe my work in identifying the limitations of a human *in vitro* neuronal aging model for studying REST function. In chapter 4, I describe my findings related to post-translational mechanisms of REST regulation in human cells. Chapter 5 is a final summary of my dissertation work and proposes future directions.

1.1 The discovery of the REST repressor

An unprecedented new mechanism of neuronal gene regulation involving a transcriptional repressor protein, was first proposed by Maue and colleagues (Maue et al., 1990). They studied the regulation of the type II sodium channel gene, Nav1.2, which is widely expressed in the nervous system and responsible for the propagation of action potentials. To this end, they isolated the genomic portion of the Nav1.2 gene consisting of 5' flanking sequences relative to the promoter from rat PC12 cells, a neuron-like cell line. Upon comparing the isolated 5' flanking sequences with that of other neuronal genes, such as peripherin and neurofilament, the authors identified a highly homologous element which shared ~86% identity with a 14bp sequence upstream of the Nav1.2 gene, including a 5bp CCAGG motif in all three genes. The most interesting observation was from a functional analysis of this isolated fragment, in which it was fused to a chloramphenicol transferase (CAT) reporter and transfected into neuronal and non-neuronal cell lines. The CAT reporter activity was 10-100 fold higher in neuronal cells compared to non-neuronal cells, suggesting a repressor mechanism likely prevented the expression of the Nav1.2 gene specifically in non-neuronal cells. This fragment was explored in more detail in a following study by Kraner and colleagues (Kraner et al., 1992). Using a similar CAT-based

reporter system and deletion analysis, the authors narrowed down the precise site of repressor activity to a striking 28bp long element upstream of the Nav1.2 gene. This region, termed as the Repressor Element 1 (RE1) was protected from digestion in DNAase footprinting assays, further revealing its role in binding to a silencer factor. In non-neuronal cells transiently transfected with of a fusion gene consisting a single copy of RE1 exhibited low CAT activity, while CAT activity driven by a minimal promoter of the Nav1.2 gene was robust. Similarly, co-transfection of the minimal promoter with 40-fold molar excess of the RE1 sequences acting as a decoy, resulted in considerable de-repression of the minimal promoter activity in non-neuronal cells. These findings taken together indicated the presence of a cell-type specific factor involved in silencing neuronal genes in non-neuronal cells. At the same time, in an independent lab, these findings of a large (21-bp) cis-acting silencer element in the Nav1.2 gene were confirmed and also identified upstream of another neuronal gene, SCG10, in this case termed as the Neuron Restrictive Silencer Element (NRSE) (Mori et al., 1992). This led to the idea that the RE1 site might regulate the expression of multiple neuronal genes in non-neuronal cells. Indeed, other investigations published results in support of this idea, following the discovery of the RE1 site upstream of more neuronal genes such as Synapsin1 (Li et al., 1993), dopamine beta-hydroxylase (Ishiguro et al., 1993), β-2 subunit of nicotinic acetylcholine receptor (Bessis et al., 1995), neuronal cell adhesion molecule Ng-CAM (Kallunki et al.,1995), and the quest to identify the cell-type specific factor binding to the RE1 site had begun.

Over the next few years, two research groups, namely the Mandel and Anderson labs, set out to identify the RE1 binding factor. Both labs had previously, independently, demonstrated evidence of RE1-binding complexes, by showing the mutation of RE1 site upstream of the Nav1.2 or SCG10 genes failed to act as competitors of RE1 binding activity *in vitro*, assessed by mobility shift assays (Kraner et al., 1992; Mori et al., 1992). Subsequently, both groups identified the RE1 binding factor at the same time using

different approaches, with the Mandel and Anderson labs naming it the RE1-Silencing transcription factor (REST) and Neuron Restrictive Silencer Factor (NRSF), respectively (Chong et al., 1995; Schoenherr & Anderson, 1995). The Mandel lab study used a one-hybrid screening method to identify the repressor protein (the prey) interacting with the RE1 site (the bait sequence) which was fused upstream to yeast reporter genes. Complementary DNA (cDNA) from HeLa cells were used to produce the hybrids between the prey and the bait. The authors isolated three yeast colonies that showed strong reporter response and performed sequence analysis on the cDNAs from these colonies. Further screening identified the open reading frame (ORF) encoding the entire REST protein. Lastly, they generated antibodies against REST and confirmed its presence in RE1 sequence complexes using gel retardation assays (Chong et al., 1995). The Anderson lab generated a probe containing three copies of the RE1 sequence of the Nav1.2 gene and screened for binding against a cDNA expression library from HeLa cells. The cDNA identified to interact with the RE1 probe was sequenced and thus revealed to encode NRSF. The authors confirmed the presence and absence of the NRSF transcripts in non-neuronal and neuronal cells, respectively, using RNase protection assays. Lastly, they also confirmed expression of NRSF in RE1 complexes with a polyclonal antibody against recombinant NRSF in vitro using electrophoretic mobility shift assays (EMSA) (Schoenherr & Anderson, 1995). Both studies showed reciprocal expression patterns of REST and neuronal gene transcripts (namely Nav1.2 and SCG10) in the developing mouse embryos using in situ hybridization, indicating REST as a factor responsible for silencing neuronal genes in non-neuronal cells during vertebrate development.

Interestingly, at the same time, a surprising discovery of REST was made in the immune system by Scholl and colleagues (Scholl et al., 1996). The authors identified a factor termed as the X2 box repressor (XBR), binding to a 19-bp silencer element, identical to the RE1, upstream of DPA, the immune-system specific major histocompatibility

complex class II (MHC-II) gene, responsible for its repression in terminally differentiated B-cell lineage. These surprising findings suggested that REST could control cell-type specific genes in different tissue systems. A key difference between REST regulation of genes in the immune vs nervous system, is in the former, REST control of MHC-II genes underlies immune-cell specification, whereas in the latter, REST restricts the expression of neuronal genes to the right cells at the right time during development and hence serves to amplify the difference between neuronal vs non-neuronal cells. Concurrently, in cells outside the nervous system, which do not give rise to neurons, neuronal genes have to be kept permanently silenced by REST. How does REST execute these silencing mechanisms in non-neuronal cells both within and outside the nervous system? The answer to this question came from first studying the functional domains in REST protein, described in the next section.

1.2 Overview of REST functional domains and interactions

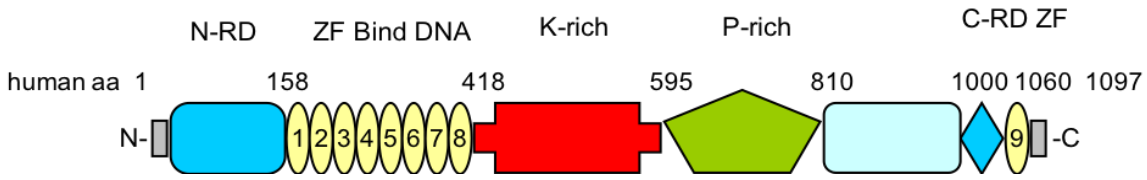


Figure 1. Schematic of human REST protein with domains required for its function as a transcriptional repressor (Illustration credit: Dr. Michael Spinner, Mandel Lab)

N-RD: N-terminal repressor domain, ZF: Zinc fingers that bind DNA, K-rich: Lysine rich region, P-rich: Proline rich region, C-RD: C-terminal repressor domain.

REST is a zinc finger protein consisting of nine zinc fingers, eight of which comprise the DNA-binding domain (Chong et al., 1995). To test whether the zinc fingers were the DNA binding entities, Chong et al over-expressed them in skeletal muscle cells, which competed with and prevented the binding of endogenous REST to the RE1 site of the Nav 1.2 gene, and resulted in de-pression of this gene. This key observation implied the DNA-binding and the repressor domains in REST were physically distinct. Indeed, the two distinct amino and carboxy-terminal repressor domains in REST (i.e. N- and C-terminal domains respectively, Refer Figure 1 schematic) were identified in a following study, using a Gal4/UAS-based genetic approach. The involvement of these two domains in target gene repression was identified from expressing REST cDNAs in PC12 cells along with CAT reporter genes containing the RE1 sites. Each of the two REST repressor domains were independently fused in-frame to the coding sequence of the DNA-binding domain in the Gal4 gene. By engineering RE1 sites in yeast UAS, and following its co-transfection with the fused Gal4 cDNAs in PC12 cells, the activity of the different REST cDNAs binding to its target RE1 sites were monitored. Through these elegant experiments, the N- and the C-terminal domains in REST were discovered to be independently sufficient in causing gene repression. Later, the ninth zinc finger in the C-terminal domain (Refer Figure 1 schematic) was also shown to be required for gene repression, which when point-mutated abrogated gene repression by the C-terminal domain in REST (Tapia-Ramírez et al., 1997). A different study published at the same time, also identified and characterized the N- and C-terminal repression domains in REST using a similar approach in a neuroblastoma cell line (Thiel et al.,1998). These discoveries of REST as a modular protein with distinct repressor domains was the first step towards understanding repressor mechanisms in mammalian cells.

By this time, yeast-based studies were beginning to show evidence of a transcriptional repressor model involving repressors binding to co-factor proteins, such as

Swi-Independent 3A or Sin3A, which in turn recruits histone deacetylases (HDACs) to the nucleosomes (Alland et al., 1997; Hassig et al., 1997; Laherty et al., 1997). Subsequently, multiple studies demonstrated Sin3A and HDAC interaction with REST at its N-terminal repressor domain using *in vitro* pull-down or immunoprecipitation assays (Huang et al., 1999; Naruse et al., 1999; Roopra et al., 2000). In all of these studies, chemical inhibition of HDAC activity by Trichostatin-A (TSA) caused relief in the repression of neuronal genes, such as GluR2 and Nav1.2, by the N-terminal REST domain. Similarly, another co-repressor protein, CoREST, was discovered to be recruited by REST at its C-terminal repressor domain using a yeast two-hybrid genetic screen (Andres et al., 1999). The C-terminal portion of REST was used as a bait to screen cDNA libraries of Gal4 activation-domain from HeLa cells. The cDNA identified from this screen was found to encode a novel 66kDa protein, which was named CoREST, that interacted with REST at its C-terminal motif containing the ninth zinc finger. REST recruitment of Co-REST, which in-turn, can also recruit HDACs, was shown to be required for REST mediated target gene repression by Ballas and colleagues (Ballas et al., 2001). Here, they introduced LacZ reporter gene with an upstream UAS sequence with Gal4-fused C-terminal REST regions, using single-cell microinjection methods along with antibodies for REST, CoREST and HDAC2 in rat fibroblasts. Presence of CoREST or HDAC2 antibodies relieved repression by the Gal4-fused C-terminal REST region as detected by an increased activity of LacZ reporter (i.e. X-gal blue product). CoREST was later identified as one of the largest co-repressor hubs in mammals, and was found to recruit several other co-factors and chromatin modifiers to REST, such as histone demethylase KDM1A (Lee et al., 2005), histone methyltransferase SUV39H1, methyl CpG binding protein 2 (MeCp2) (Lunyak et al., 2002), and members of SWI/SNF chromatin remodeling complexes (Battaglioli et al., 2002).

To summarize, REST repression of its neuronal gene targets is mediated by two distinct repressor domains at its N- and C-terminal regions. Repression from the N- and C-terminus is mediated through its interaction with Sin3A, and Co-REST, respectively. Both Sin3A and CoREST in turn recruit histone deacetylases, namely HDAC1/2. Additionally, CoREST can further recruit a host of other chromatin remodeling factors such as KDM1A, MeCP2, SUV39H1, and members of the SWI/SNF complex. Together, this entire REST repressor complex through epigenetic remodeling allows for repression of neuronal genes (Refer Figure 2 schematic).

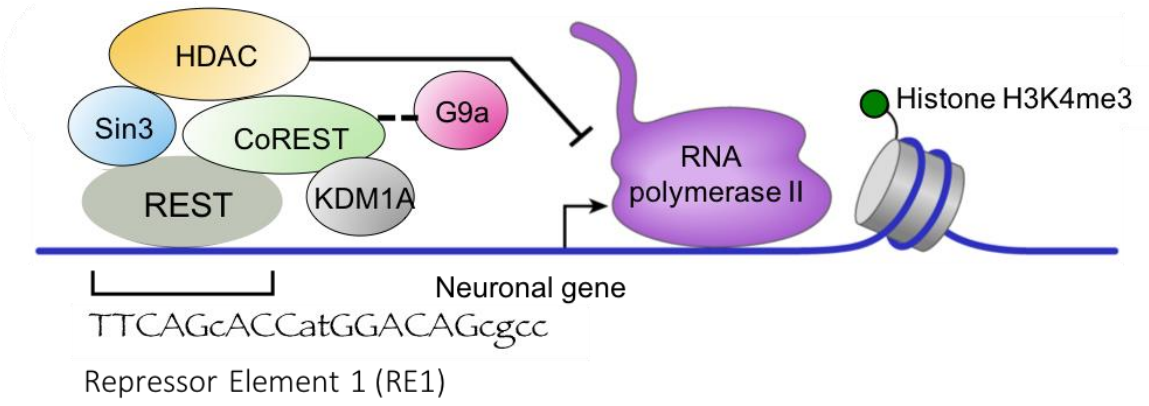


Figure 2. Simplified schematic of REST repressor complex facilitating neuronal gene repression through chromatin remodeling (Illustration credit: Mandel lab)

1.3 Role of REST in mammalian neurogenesis

As a negative regulator of neuronal genes, REST plays crucial roles in maintaining the identity of non-neuronal cells outside the nervous system as well as restricting neuronal gene expression to neurons during vertebrate neurogenesis. These distinct roles for REST was first described by Chen and colleagues (Chen et al., 1998). In their study, they generated global REST knockout (REST -/-) mice, which unexpectedly, did not

survive beyond embryonic day 11.5 (E11.5). However, two interesting observations were made in this work. First, REST was shown to be ubiquitously expressed until E9.5. Second, upregulated expression of βIII tubulin, a neuronal gene containing RE1 sequences capable of REST binding, was detected in non-neuronal tissues of REST -/- embryos by E9.5. In the same study, they introduced a dominant-negative REST mutant in chicken neural tube, targeting neural progenitors and crest cells, which resulted in the de-repression of neuronal genes such as SCG10, Ng-CAM and βIII tubulin, leading to their precocious expression during development. Together, these observations confirmed in vivo REST repression of neuronal genes in non-neuronal cells outside the nervous system, as well as in neural progenitors, and suggested that REST likely regulated the timing of neuronal gene expression during development. Many years later, it was a seminal study by Ballas and colleagues which provided further insights into the developmental regulation of REST (Ballas et al., 2005). Using an *in vitro* neuronal differentiation model, the authors showed that REST was downregulated at two distinct stages. They utilized mouse embryonic stem cells (mESCs), which were differentiated into post-mitotic neurons via neural progenitors using retinoic acid. mESCs expressed high levels of REST, which was bound to RE1 sites in neuronal genes, such as Calbindin D28K, along with co-repressors CoREST and HDAC. During transition of mESCs to neural progenitors, REST protein was rapidly degraded through the proteasome. Surprisingly, despite this global loss of REST protein in the progenitors determined by the absence of REST in western blotting assays, REST was still bound to neuronal gene chromatin, detected by quantitative chromatin immunoprecipitation (qChIP) assays. However, prior to the transition of the progenitors to a post-mitotic stage, REST and its entire co-repressor complex (eg.,CoREST, HDAC, Sin3) was removed from its binding to RE1 sites in neuronal genes, which was essential for the induction of neuronal gene expression in the

post-mitotic neurons. Further insights into the in vivo role for REST during early neuronal development was provided by Mandel and colleagues (Mandel et al., 2011). Here, they overexpressed REST during neocortical development in mice by in utero electroporation. This prolonged expression of REST blocked migration of neural progenitors, causing their arrest at the boundary of the ventricular/sub ventricular zone and the intermediate zone leading to a delayed transition to a neuronal state. However, since the cells eventually became neurons, it was concluded that REST acted as a timer for terminal neuronal differentiation, but was not critical for neuronal fate acquisition in vivo. The functional consequences of disrupting this timely differentiation of neurons by REST in vivo was demonstrated in a subsequent study by Nechiporuk et al (Nechiporuk et al., 2016). They generated mice with a conditional knockout of REST in neural progenitors, prior to the time REST is normally removed from chromatin during neurogenesis, using a gene trap approach. This premature loss of REST from neural progenitors caused DNA damage during the S phase of cell division and early cell cycle exit, accompanied by a depletion of progenitors and apoptosis resulting in smaller brains compared to that of wildtype mice.

To summarize, REST represses neuronal genes in non-neuronal cells outside the nervous system as well as in neural progenitors. REST needs to be progressively downregulated during *in vitro* neuronal differentiation to allow for the elaboration of a mature neuronal phenotype. During in-vivo neurogenesis, the precise timing of REST removal from the progenitors ensures proper terminal neuronal differentiation. In the next section, I will describe the various mechanisms that control REST expression, which in-turn is key to regulating its activity in the formation and maintenance of neurons.

1.4 Regulation of REST expression

1.4.1 Post-transcriptional control of REST expression

Post-transcriptional regulation of genes is an important mechanism of gene expression control at the RNA level. It can occur at different stages such as alternative splicing, control of RNA stability and nuclear export. This chapter will describe microRNAs, RNA-binding proteins and alternative splicing mediated control of REST expression. In particular, the regulatory relationship between REST and microRNAs provides relevant background for the discussion section of my work described in Chapter 3.

1.4.1.1. Regulation of REST expression by MicroRNAs

MicroRNAs (miRNAs) are small (21-25 nucleotide) non-coding RNAs that post-transcriptionally regulate gene expression in a sequence specific manner, by targeting mRNAs for translational repression or degradation (He & Hannon, 2004). Generation of miRNAs begins with a primary-miRNA transcript, which is then processed into a stem-loop precursor-miRNA that is ultimately transported to the cytoplasm to produce the mature and functional miRNA (O'Brien et al., 2018). This mature miRNA can be generated from both 5' and 3' strands of the stem loop precursor-miRNA, with one species more predominantly produced than the other. In such cases, the less predominant mature miRNA will be appended with an asterisk (*) to differentiate between the two species of mature miRNA (Hammond, 2015).

Computational studies first predicted miRNA recognition elements (MRE) in REST mRNAs (Wu & Xie, 2006). However, the first evidence showing REST as a bona-fide target of miRNAs, namely miR-9, was demonstrated by Packer and colleagues (Packer et al., 2008). miR-9/9* is one of the most abundant miRNAs found in the mammalian brain. Using luciferase reporter-based assays the authors demonstrated a functional impact of

miR-9/9* on REST mRNA levels. Specifically, co-transfection of luciferase reporter constructs consisting the 3' UTR of REST with varying amounts of miR-9/9* in human embryonic kidney cells (HEKs) resulted in a dose-dependent decrease in luciferase activity. Further, in order to ascertain whether this effect was due to miR-9 or miR-9* or both, the authors co-transfected the 3' UTR REST-luciferase constructs with either pre-miR-9 or pre-miR-9* and monitored luciferase activity. Interestingly, only miR-9 significantly reduced luciferase activity, demonstrating REST was targeted predominantly by miR-9. Conversely, co-transfection of antisense sequences to specifically block miR interactions in REST mRNAs, rescued the effect of miR-9/9* on luciferase activity. Together, these findings demonstrated REST mRNA as a functional target of miR-9. Interestingly, this study also showed a similar functional effect of miR-9* on CoREST mRNA levels, suggesting miR9/9* mediated regulation of the REST repressor complex. A later study by Giusti and colleagues, showed the molecular implications of REST downregulation by miR-9 in dendritic development (Giusti et al., 2014). Here, they utilized miRNA-9 sponges, which are RNA transcripts consisting of binding sites for miRNA-9, as a dominant negative tool to eliminate miR-9 function. As expected based on previous studies, miR-9 sponge expressing mouse hippocampal neurons exhibited increased REST mRNA levels. Further, these neurons had reduced dendritic length and complexity compared to control (i.e. sponge-less) neurons. Overexpression of REST, in miR-9 sponge expressing hippocampal neurons rescued these dendritic effects, indicating the dendritic phenotypes with loss of miR-9 was mediated by REST. Interestingly, unlike REST, overexpression of other miR-9 targets such as Foxp4, Creb1 and Map1b, did not rescue these dendritic defects, suggesting REST as the main miR-9 target to be involved in regulating dendritic growth. Lastly, transfection of short hairpin RNA (shRNA) to downregulate REST in neural precursors of transgenic miR-9 sponge mice rescued the dendritic defects, confirming increased REST activity underlied dendritic impairment.

While the above studies demonstrate REST regulation by miRNAs, a reciprocal mechanism i.e. regulation of miRNAs by REST has also been shown. A study from members of the Mandel lab discovered miR-124a, the predominant mature form of miR-124, and miR-9/9*, both highly expressed miRNAs in the mammalian brain, to be regulated by REST (Conaco et al.,2006). They first identified and validated REST binding in close proximity to miR-124a and miR-9/9* genes by serial analysis of chromatin occupancy (SACO) and chromatin immunoprecipitation (ChIP) assays. Expression of a dominant negative REST construct without its repressor domains in mouse embryonic fibroblasts increased levels of miR-9/9* and -124, confirming REST regulation of these miRNAs. Further, reciprocal expression patterns of REST and miR-124a was observed during *in vitro* neuronal differentiation of mouse embryonal carcinoma (P19) cells. Specifically, upregulation of miR-124a in the mature neurons coincided with a loss of REST levels. ChIP analysis in the mature neurons confirmed dismissal of REST from its target site in the miR-124a gene, thus identifying loss of REST as an underlying mechanism for increased miR-124a levels. Blocking miR-124a in mature neurons using antisense oligonucleotides resulted in increased levels of non-neuronal gene transcripts, such as Caveolin-1, indicating that REST repression of miR-124a allowed for the expression of non-neuronal genes and thus relieving REST repression of miR-124a during neuronal differentiation established a neuronal phenotype through suppression of non-neuronal genes.

Lastly, REST has also been implicated in regulating long non-coding RNAs (lncRNAs) (Johnson et al., 2009), however this has remained largely unexplored.

1.4.1.2. Regulation of REST expression by RNA-binding proteins

While REST is downregulated at terminal neuronal differentiation, paradoxically it is still expressed in post-mitotic neurons of the adult nervous system, albeit at low levels. Given that REST silences neuronal genes, regulation of its levels in mature neurons is key to ensuring neuronal gene expression. A study by Cargnin and colleagues, identified a post-transcriptional mechanism by which REST levels were regulated in mature mouse peripheral neurons (Cargnin et al., 2014). Using an unbiased yeast three hybrid screen, the authors discovered an RNA-binding protein, zinc finger 36-like 2 (ZFP36L2), as a potential candidate to regulate REST mRNA levels. They functionally validated that ZFP36L2 targets REST at a site within its 3' UTR region using luciferase-based assays and also confirmed direct binding of ZFP36L2 to REST 3' UTR using gel shift assays in PC12 cells. In cultured neurons from superior cervical ganglia (SCG) and dorsal root ganglia (DRG) of ZFP36L2 knockout mice, elevated REST mRNA (>3 fold) and protein were detected, along with decreased expression of REST regulated neuronal genes, such as SNAP25 and Nav1.2, indicating increased repressor function of REST in the absence of ZFP36L2. A functional consequence of increased REST levels in ZFP36L2 knock out mice were defects in axonal integrity/outgrowth observed in DRG organotypic cultures. Knockdown of REST in the ZFP36L2 knockout DRG organotypic cultures using shRNA rescued the defects in axonal integrity, implicating REST regulation by ZFP36L2 in the maintenance of axonal health. Together, these findings demonstrated that a post-transcriptional mechanism of REST control by RNA-binding proteins, such as ZFP36L2, were important for the maintenance of axonal health in mature mammalian peripheral neurons.

1.4.1.3. Alternative splicing control of REST expression

The first step in eukaryotic gene transcription is the generation of a primary mRNA transcript, known as the pre-mRNA, consisting of protein coding (exons) and non-coding (introns) sequences. The pre-mRNA is then subject to alternative splicing, a process by which entire or portions of coding or non-coding regions are differentially joined or skipped, resulting in multiple mature mRNAs (Matlin et al., 2005). These mRNA splicing isoforms can each give rise to a protein, thereby resulting in varied protein isoforms differing in structure and function produced from a single gene.

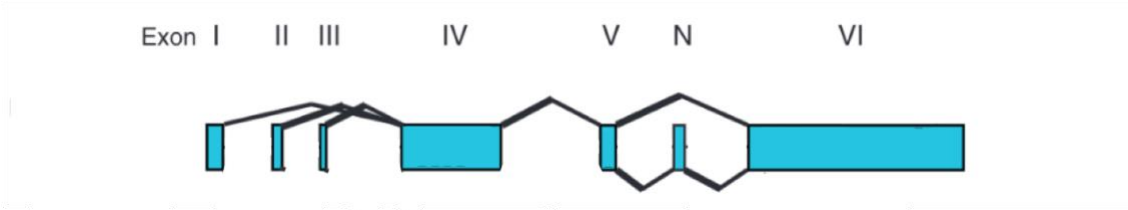


Figure 3. Illustration adapted from Aigner & Yeo, 2009. Structure of the REST gene depicting the alternate isoforms. Exons are indicated as boxes and numbered from I – VI as shown above the respective exons. Exons I-III are alternative 5' UTRs that are spliced to exon IV, represented by the lines connecting them.

The first study to show evidence of alternate splice variants in REST was by Palm et al. in the adult rat brain (Palm et al., 1998). They identified alternate splice sites for exons V, N and VI in REST by PCR based analysis (Refer Figure 3 schematic), which predicted truncated forms of REST protein. A detailed examination of four REST splice variants by RNAse protection assay revealed rat brain-specific expression for two of the variants, while the remaining two were ubiquitously expressed in the brain and other rat body regions such as thymus, heart, lung, to name a few. They also estimated the relative abundance of these four splice variants by RT-PCR in the rat brain, which revealed

predominantly high levels of one variant predicted to contain all the nine zinc fingers in REST. The functional consequences of two truncated REST splice variants on REST target gene expression was explored in Neuro-2A cells using a CAT-based reporter gene system. Both isoforms of REST, containing either four (REST1^{trunc}) or five (REST2-5^{trunc}) zinc fingers were capable of target gene repression, with REST1^{trunc} exhibiting higher levels of repression compared to REST2-5^{trunc}, indicating potential functional differences in the different REST isoforms.

Many years later, a study by Raj and colleagues discovered a REST splicing factor, known as the Ser/Arg Repetitive Matrix 4 (SRRM4), which activated the inclusion of exon N (Refer Figure 3 schematic) (Raj et al., 2011). Inclusion of exon N created a frameshift and introduced a stop codon at the beginning of exon VI, resulting in a truncated isoform of REST, referred to as REST4, consisting of only the N-terminal repressor domain and the first five zinc fingers. REST4 was demonstrated to be a neural-specific isoform, based on its predominant expression in neural cell lines, such as Neuro2a, as opposed to non-neuronal cell lines, such as NIH-3T3 fibroblasts. Knockdown of SRRM4 in Neuro2a cells resulted in increased skipping of exon N, suggesting direct regulation of REST alternative splicing by SRRM4, which was further validated using UV crosslinking immunoprecipitation (CLIP) assays. An important finding, was that the knockdown of SRRM4 in the developing mouse cortex at E13/14 by in-utero electroporation of shRNAs, caused migration defects in the precursor cells from the ventricular/sub-ventricular zone to the cortical mantle. Additionally, a reduction (3-fold) in the number of cells committed to a neuronal fate, based on the fraction of cells expressing βIII-tubulin, a REST target gene, and the fraction of cells expressing precursor marker Pax6, suggested that a prevention of REST alternative splicing by SRRM4 may cause developmental defects, specifically in the transition of precursor cells to neurons. While the findings from this study implicates a role for the REST4 isoform, how it affected REST function during neuronal

differentiation was not evaluated. This is important to know, because, prior studies aimed at characterizing REST4 function *in vitro* in a neuronal cell line, found that REST4 does not act as a transcriptional repressor, neither does it act as a de-repressor of REST-mediated transcriptional repression (Magin et al.,2002). However, these results are also controversial since they were performed in a cholinergic cell line, NS20Y, which may not truly represent all properties of neurons (O'Bryant et al., 2016).

By this time, other studies in the field had identified new REST splice variants with skipped exon IV and/exon V, (Refer Figure 3, schematic of REST gene structure), using nested PCRs from cDNA of normal human tissue and cancer cell lines (Chen & Miller, 2013). Interestingly, an analysis of REST splice variants in non-human primate tissue and mouse hippocampus revealed predominant skipping of exon V and exon IV alone, respectively, suggesting species-specific differences in REST isoforms. The REST protein domains encoded by exon V includes the fifth zinc finger of the DNA-binding domain which has a nuclear localization signal (Shimojo, 2006). Hence, skipping exon V could impact REST translocation into the nucleus, thereby reducing its capacity to function as a transcriptional repressor. Indeed, this has been demonstrated in the context of Huntington's disease (HD) (Chen et al., 2017). In a cellular model of HD, Chen and colleagues showed that causing skipping of exon V in REST using anti-sense oligonucleotides (ASOs) reduced nuclear levels of REST. This, in turn, caused de-repression of REST regulated neuronal genes such as Synapsin1 and STMN2, whose repression had previously been implicated in causing neurotoxicity in HD (Buckley et al.,2010). Thus, using ASOs to facilitate the alternate splicing of REST exon V in HD was proposed as a potential therapeutic option to alleviate neurotoxicity in HD.

Lastly, a more recent study evaluated the contribution of SRRM4-mediated alternate splicing of REST to the REST4 isoform, in adult mice, in the context of hearing (Nakano et al., 2018). Here, Nakano and colleagues showed that inclusion of REST exon

N by SRRM4 was important for proper functioning of adult mechanosensory hair cells in mice. Transgenic mice that lacked the inclusion of exon N in REST showed degeneration of hair cells by P12, deafness and balance defects compared to mice that had the exon N inclusion in REST. Moreover, an intronic mutation (C > G) upstream of exon N, identified as a deafness-associated variant in humans (DFNA27), prevented the proper alternative splicing of exon N by SRRM4, resulting in an aberrant spliced REST isoform. Using mini-genes and luciferase assays, the authors demonstrated that the aberrant splicing due to the C>G mutation generated active forms of REST protein in the presence of SRRM4, capable of repressing several hearing and balance related genes. Thus, an alternative splicing event in REST was shown to be critical for REST inactivation in hair cells, and defects in this mechanism, such as due the presence of a C>G mutation upstream of exon N in REST, can cause deafness.

In summary, there is strong evidence of several REST splice variants, with potentially varied functions in different cellular contexts. However, only few of the corresponding protein isoforms have been experimentally validated and assessed for functionality. It is also likely many of these protein isoforms are subjected to modifications at the post-translational level, reviewed in the next section, making it more challenging to study their function.

1.4.2 Post-translational control of REST expression

Similar to the genome, a vast degree of diversity is present in the proteome. This increased complexity is facilitated by post-translational modifications (PTMs). As the name suggests, PTMs refers to chemical modifications added to proteins after their bio-synthesis. These modifications can change the properties of their target proteins often resulting in changes to their activity, localization and interaction with other cellular

molecules (Ramazi & Zahiri, 2021). This chapter will detail the discovery of PTMs in REST and their influence on REST function and serves as the background for my work described in Chapter 4.

1.4.2.1. Ubiquitination of REST

Ubiquitination refers to the process by which ubiquitin, a small regulatory protein is covalently linked to a target protein. While canonical ubiquitination occurs at lysine residues (eg., K6, K11, K27, K29, K33, K48), non-lysine residues (eg., serine, threonine, cysteine) have also been reported to be sites of ubiquitination (Mcclellan et al., 2019). One of the major functions of protein ubiquitination is to facilitate its degradation through the ubiquitin proteasome system (UPS), a highly specific intracellular pathway consisting of various molecular components responsible for the targeted degradation of proteins (Ciechanover & Schwartz, 1998). This targeted conjugation of ubiquitin to proteins can be mono or poly in nature, depending on the number of ubiquitins attached to a single substrate protein and is mediated by specific enzymes known as E3 ubiquitin ligases, such as the Skp1-cullin1-F-box (SCF) proteins (Zheng & Shabek, 2017). SCF is the largest E3 ubiquitin ligase family and a multi-component complex consisting of three core subunits (Skp1, Cullin1 and RBX1) and a variable F-box protein (Zheng et al., 2002). The F-box protein is the subunit that mediates the interaction between the SCF complex and its target protein, thereby acting as the substrate protein recognition component of the SCF complex. There are at least 38 known F-box proteins in humans, one of which is β-TRCP (Kipreos & Pagano, 2000).

Two studies, independently, discovered ubiquitination of REST mediated by its interaction with β-TRCP, leading to its degradation through the UPS (Guardavaccaro et al., 2008; Westbrook et al., 2008). Each of these two studies identified one β-TRCP

interaction site each within the C-terminal domain of REST in mammalian cells using different approaches (Refer Figure 4 schematic). In the study by Westbrook and colleagues, a fluorescence based high throughput *in vitro* screen was developed to identify F-box proteins responsible for REST degradation. They overexpressed REST fused with a constitutively active red fluorescence protein (mRFP) in human embryonic kidney (HEK) cells and monitored REST-mRFP levels following treatment with siRNAs targeting different F-box proteins of the SCF family, leading to the identification of β-TRCP. Frame-shift mutation in REST resulting in an altered C-terminal domain, prevented β-TRCP recruitment and increased stability of REST protein, suggesting the site of interaction to be located in the C-terminal domain of REST. Using mass spectrometry, they further delineated this interaction site to a conserved degron sequence in REST (Refer Figure 4 schematic). Lastly, expression of degron-mutant REST in mESCs caused a significant suppression of its neuronal differentiation *in vitro*, compared to the expression of wild-type REST, implicating β-TRCP mediated REST degradation to be critical for neuronal fate acquisition. In the other study by Guardavaccaro et al, the authors purified ubiquitinated substrates of F-box proteins and performed an unbiased mass spectrometry analysis in order to identify substrate proteins targeted by β-TRCP-SCF complex. This analysis revealed a direct interaction between β-TRCP and REST, and identified a second degron sequence in C-terminal REST domain to be the site of interaction (Refer Figure 4 schematic). Recruitment of β-TRCP by REST was required for its degradation during the G2 phase of mitosis in HeLa cells, which in-turn allowed for de-repression of its target gene, Mad2, which controls the transition from metaphase to the anaphase stages of mitosis. Expression of REST mutated at the β-TRCP recruitment site in HCT116 cells, a cancer cell line, prevented Mad2 gene upregulation resulting in chromosomal aberrations indicative of premature anaphase transition.

While ubiquitination of REST by β-TRCP facilitates its degradation, a reciprocal mechanism known as deubiquitination stabilizes REST protein. Protein deubiquitination is catalyzed by deubiquitinating enzymes, which cleave the mono- or poly-ubiquitin chains from proteins, thereby preventing proteolysis through the UPS (He et al., 2016). REST de-ubiquitination was reported in a study by Huang and colleagues (Huang et al., 2011), which identified herpesvirus-associated ubiquitin-specific protease (HAUSP), a deubiquitinating enzyme, to be recruited by REST at the PYSS site within its DNA binding domain (Refer Figure 4 schematic). The functional significance of REST deubiquitination was demonstrated in neural progenitor cells (NPCs), where knockdown of HAUSP in NPCs reduced REST levels leading to disrupted proliferation of NPCs and premature induction of neuronal differentiation. Conversely, overexpression of HAUSP prevented β-TRCP mediated REST degradation during neuronal differentiation. These findings are significant because they demonstrate that β-TRCP-mediated ubiquitination and HAUSP-mediated deubiquitination of REST are critical post-translational control mechanisms that determine the maintenance of NPCs vs their differentiation into neurons.

1.4.2.2 Phosphorylation of REST

Protein phosphorylation is a widespread PTM which involves the addition of phosphate groups to specific amino acid residues. This process is catalyzed by protein kinases, typically at serine, threonine or tyrosine amino acid sites. Addition of phosphate groups alters the charge of the substrate protein, leading to a change in conformation and function (Nestler & Greengard, 1999). Following the discovery of the β-TRCP-recruiting degrons in REST protein, a study by Nesti and colleagues (Nesti et al., 2014) identified a new phosphorylation site in REST at serines S861/S864, catalyzed by proline-directed kinases ERK1/2, upstream of the two β-TRCP-binding sites (Refer Figure 4 schematic).

Preventing phosphorylation at this site stabilized REST levels by preventing its degradation through the UPS in HEK cells, suggesting an interplay between REST phosphorylation and ubiquitination in regulating its levels. Indeed, in HEKs transfected with REST mutated at S861/S864 that prevented its phosphorylation, β-TRCP was not recruited, as detected by immunoprecipitation assays. Lastly, transfection of cDNA encoding a portion of REST containing S861/S864 and the β-TRCP binding sites in mouse neurospheres stabilized endogenous REST by acting as a degradation signaling decoy. However, expression of the same REST construct with mutated S861/S864 sites failed to stabilize endogenous REST. Consequently, neurospheres with stabilized endogenous REST were inhibited in their ability to differentiate into neurons. Together, these findings demonstrated the requirement of REST phosphorylation at S861/S864 in its timely degradation by β-TRCP during neuronal differentiation. Since phosphorylation is a reversible modification, dephosphorylation of REST by a phosphatase, should have an opposing effect on REST protein levels. The same study demonstrated the role of a phosphatase, namely CTDSP1 (C-terminal domain RNA polymerase II polypeptide A small phosphatase 1), previously known to be recruited by REST to neuronal genes, in stabilizing REST levels in HEKs. Co-transfection of HEKs with CTDSP1 and REST stabilized REST levels, detected by western blotting assays. More recently, a different study showed that the knock down of endogenous CTDSP1 in REST expressing HEK cells resulted in reduced REST levels, leading to upregulated expression of REST target neuronal genes such as βIII-tubulin and BDNF. Based on these findings, and the previous results from Nesti et al., the authors proposed a model where dephosphorylation of REST at S861/S864 by CTDSP1 prevented its targeted degradation by β-TRCP, thereby stabilizing its levels and sustaining its repression of neuronal genes in HEKs (Burkholder et al., 2019).

1.4.2.3. Methylation of REST

Protein methylation refers to the addition of a methyl group (-CH_3), most commonly to the nitrogen containing side-chains of arginine and lysine amino acid residues. Substrate proteins can generally accept upto three methyl groups, resulting in mono-, di- or tri-methylated residues, catalyzed by enzymes known as methyltransferases. A recent study reported methylation of REST protein, for the first time, at a site within its lysine-rich domain (K494) in human cells (Lee et al., 2018). This PTM on REST was found to be mediated by a methyltransferase, known as, the enhancer of zeste homolog 2 (EZH2).

EZH2 has been extensively studied in regards to its role as a catalytic subunit of the polycomb repressive complex (PRC2), an epigenetic regulator, that has important roles during mammalian development. As part of the PRC2, EZH2 catalyzes methylation of histone H3 at lysine 27 (H3K27Me3) to silence gene transcription (Wassef et al., 2019). While the majority of studies on EZH2 focused on its canonical role within PRC2, recent studies are beginning to uncover PRC2-independent non-canonical roles for EZH2 (Kim et al., 2013; Lee et al., 2012; Wang & Wang, 2020). The proposed methylation of REST by EZH2 is also thought to be a non-canonical function of EZH2, independent of its role in the PRC2. In their study, Lee and colleagues first identified a direct interaction between REST and EZH2 by co-immunoprecipitation in human dermal fibroblasts and HEK cells. Further, they narrowed the site of REST methylation to lysine (K) 494, belonging to the conserved R-K-S amino acid sequence in human REST protein (Refer Figure 4 schematic), based on previous studies that showed lysine residues within a similar R-K-S sequence of amino acids can serve as the acceptor of a methyl group from EZH2 (Lee et al., 2012). The most interesting observation was that mutation of REST at K494 (K494>A) affected its stability in HEKs. Specifically, K494>A-REST had a shorter half-life compared to wild-type REST in transfected HEKs, shown by *in vitro* protein degradation assays. An

important caveat to note here, was the lack of biological replicates and statistical evaluation in the presentation of these results, raising important questions on whether the observed effect of K494 on REST stability is accurate and reproducible. Moreover, whether methylation at K494 regulated REST recruitment of β-TRCP and its subsequent degradation through the proteasome was not investigated in this study, and remains an open question. Lastly, a functional consequence of the regulation of REST levels by EZH2 via methylation was discussed in the context of the *in vitro* trans-differentiation of human dermal fibroblasts to neurons. This is described more in detail in Chapter 4.

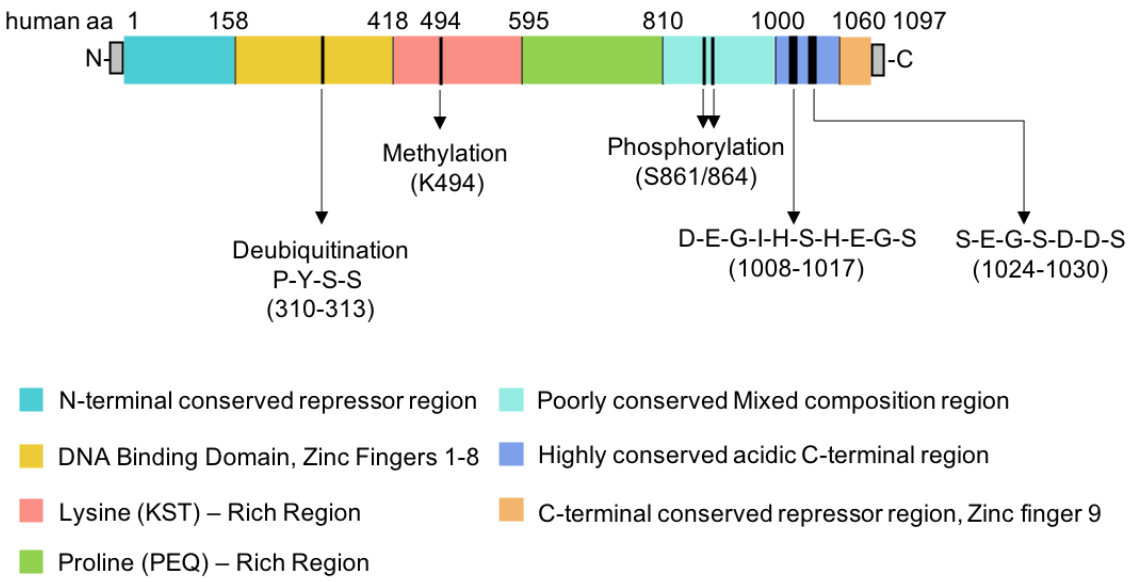


Figure 4. Schematic representation of the human REST protein to illustrate the sites identified in regulating its levels via post-translational mechanisms. The two degron sequences - D-E-G-I-H-S-H-E-G-S and S-E-G-S-D-D-S recruit the E3 ubiquitin ligase, β-TRCP.

1.5 REST in the adult brain

Historically, for over two decades, the majority of studies on REST in the nervous system was related to its role in formation of the mouse embryonic nervous system. However, more recent studies have indicated roles for REST in the adult rodent brain. Several studies have detected REST expression in the post-natal rodent brain (Palm et al., 1998; Kuwabara et al., 2005; Sun et al., 2005; Gao et al., 2011; Nechiporuk et al., 2016), including elevated REST levels under conditions of ischemia or seizure (Calderone et al., 2003; Hu et al., 2011; Kaneko et al., 2013; McClelland et al., 2014). More recent studies in the adult human brain have also detected REST expression in the mature human brain and proposed roles for REST during brain aging (Lu et al., 2014; Schiffer et al., 2014; McGann et al., 2021). This chapter will focus on the recent discoveries related to REST function in human aging and neurodegenerative diseases, such as Alzheimer's, and is relevant background for my work described in Chapter 3.

1.5.1 Role of REST in human brain aging and Alzheimer's disease

The first evidence of REST expression in the adult human brain was shown in the year 2014. Schiffer and colleagues reported expression of REST protein in different areas of archived human brain tissue, such as, cortex, caudate nucleus, hippocampus and cerebellum, using immunohistochemistry (Schiffer et al., 2014). REST expression coincided with neuronal as well as glial markers, with a more intense nuclear staining pattern in glial cells such as astrocytes and relatively more cytoplasmic expression in some neurons such as purkinje and granular layer neurons. As a transcriptional repressor, this cytoplasmic expression of REST is controversial, as there is no clear evidence of whether this non-nuclear staining is authentic or due to technical artefacts, especially since later studies have demonstrated that artificial cytoplasmic REST can be detected

under certain immunostaining conditions of cells *in vitro* (Chen & Miller, 2018). Around the same time in 2014, a different study by Lu et al identified REST expression in archived adult human prefrontal cortex (Lu et al., 2014). Importantly, this study showed an induction of REST levels in neurons of prefrontal cortex in old individuals (71-95 years), compared to that of young individuals (24-29 years), by western blotting. Interestingly, REST levels in neurons of the prefrontal cortex from aged Alzheimer's disease (AD) patients, were low, similar to that of the young healthy samples. To assess the functional consequence of reduced REST in AD brains, the authors compared the binding patterns of REST to stress-related and apoptotic genes, such as, PUMA, BAX, BID, etc., in neurons isolated from AD and age-matched healthy prefrontal cortex tissue by quantitative ChIP (qChIP). These specific set of genes were chosen to profile REST binding, based on identifying them as direct targets of REST in a human neural progenitor cell line (SH-SY5Y). While they did report significant decrease in REST binding to the stress-related and apoptotic genes in the AD pre-frontal cortex neurons, compared to healthy controls, it raises the question of whether their analysis was biased, since these genes were identified as REST targets in a progenitor cell line, and not post-mitotic neurons of the human brain. Lastly, their immunochemical analysis of REST expression in prefrontal cortex neurons of the AD brains showed reduced nuclear and more cytoplasmic REST, compared to that of the healthy controls, which is also controversial since cytoplasmic REST staining can result from technical artefacts, as later shown in *in vitro* cellular systems (Chen & Miller, 2018). Collectively, based on the above described findings, the authors proposed a neuroprotective role for REST in the aging human brain and implicated a loss of REST function in neurodegeneration underlying AD.

A major caveat of the above study was the lack of an unbiased analysis of the direct binding sites for REST in the aged healthy human brain. In order to fill this gap in knowledge, members of the Mandel lab profiled REST expression and genome-wide

binding in archived aged human brain (hippocampus) using ChIP-Seq (McGann et al., 2021). Similar to findings from Lu et al., that showed an induction of REST in the aged human brain, the authors of this study showed an increase in REST protein levels, beginning at the end of the third decade of life that remained elevated even in nonagenarians, in the human hippocampus. Surprisingly, this was in complete contrast to REST expression patterns in the mouse hippocampus, where REST levels were highest in early adulthood and declined with age in mice, suggesting novel human-specific roles for REST in the aging brain. Indeed, a comparison of genome-wide REST binding profiles between the aged human hippocampus (68y old) and the mouse hippocampus (5-weeks old), revealed differences in the total number and nature of REST binding sites between the two species. A caveat here was that 5-week old juvenile mice hippocampal samples were used for REST ChIP-Seq analyses because of the drop in REST levels during aging in mice. The authors identified significantly higher number of REST binding sites in the human hippocampus compared to that of the mouse. Additionally, a majority of REST bound genes in the human hippocampus were unique and previously unknown targets of REST belonging to the innate immune system, such as ALOX5, AMPD3, C1QA/B, COTL1, whereas most of the REST targets in the mouse hippocampus were canonical RE1 sites with very minimal overlap to that of the human hippocampus. Apart from identifying a novel set of human specific-immune gene targets of REST, these results also highlight the fact that mouse models may not be truly representative of human brain aging mechanisms. Interestingly, REST binding to the human-specific immune genes in the brain occurred at non-RE1 DNA motifs, suggesting the evolution of new binding sites for REST in humans or indirect recruitment of REST to these sites by other transcription factors. While the former hypothesis needs more evidence, the authors identified an enrichment of motifs for other immune-related transcription factors, such as Sp2, Runx1 and Gfi1b, at these non-RE1 sites, in support of the latter hypothesis. Finally, these

findings also raise the important question of the cellular basis of REST-mediated gene regulation during human brain aging. Ongoing studies by other members of our lab aim to identify the cellular source of REST binding to immune genes, i.e., whether it is in neurons, glia, others such as endothelial cells, or a combination of cell types.

Lastly, more recent studies are beginning to utilize *in vitro* human neuronal systems to gain insights into the mechanisms underlying aging disorders such as AD. Advances in the field of stem cell biology have given rise to various cellular reprogramming approaches that allow for the generation of human neurons *in vitro* from skin samples of neonates or adults, including samples from patients with AD (Ming et al., 2011). Two recent independent studies utilized distinct cellular reprogramming strategies to generate *in vitro* neurons from patients with AD. Interestingly, they discovered completely opposing patterns of dysregulation in neuronal genes that are regulated by REST, in their respective AD human neurons, alluding to opposing roles for REST in AD (Mertens et., 2015; Meyer et al., 2019). These controversial findings are described in detail in Chapter 5, where I discuss them in the context of my own findings related to using human *in vitro* neuronal systems to study REST function in aging.

Chapter 2: Materials and Methods

Direct reprogramming of human fibroblasts to neurons

Protocol adapted from Richner et al (Richner et al., 2015). Lentiviral preparations of the reverse tetracycline-controlled transactivator (rtTA; Addgene, 66810) and pT- BclXL-9/9*-124 plasmid constructs (both received from Yoo lab at Washington University in St Louis) were carried out in HEK-293T cells as previously described (Richner et al., 2015), and titer estimation was performed using Rapid lentiviral p24 titer kit (Takara Bio, #632200). Corning cell culture plates (6-well or 10cm or 12-well) were coated with Gelatin (0.1%) for 15 minutes at 37°C. Seeding density was optimized for cell culture plates of different sizes. The following day, human fibroblasts were transduced with a reprogramming cocktail of concentrated lentivirus containing the reverse tetracycline-controlled transactivator (rtTA; Addgene, 66810) and pT- BclXL-9/9*-124 (42 ul of virus at recommended titer per $2x10^5$ cells) in presence of polybrene (8ug/mL). Cells on multi-well plates were spinfected at 37°C for 30 minutes at 1000g. The next day fresh fibroblast media (prepared as per Coriell Institute's guidelines with DMEM media, Fetal Bovine Serum and penicillin-streptomycin solution) was changed with doxycycline (Dox, 1 mg/mL, Sigma D9891). Two days later, fresh fibroblast media was changed with Dox and antibiotics for selection of cells transduced with microRNAs-9/9* and -124 (Puromycin, 3 mg/mL, Life tech/Invitrogen (A11138-03)). For immunostaining, five days post-transduction cells were re-plated to acid-treated and poly-ornithine (Sigma #P4957 at 0.1 mg/mL), laminin (Sigma #L2020 at 5 ug/mL), fibronectin (Sigma #F4759 at 2 ug/mL) coated German glass coverslips (neuvitro 12mm or 25mm) using drop-plating method as previously described (Richner et al., 2015). For all other biochemical assays, cells were re-plated to Primaria plates (Corning, 10cm #353803 or 6-well #353846). The following day Neuronal media was added (BrainPhys, Stem Cell Technologies) supplemented with Dox, 1mM valproic acid (EMD Millipore, #676380), 200mM dibutyryl cAMP (Sigma-Aldrich, D0627), 10 ng/mL

BDNF and NT-3 (Peprotech, #450-02 & #450-03), and 1uM Retinoic Acid (Sigma-Aldrich, #R2625) with 3 mg/mL Puromycin. Dox was replenished every two days and half media changes was done every 4 days until the end of the reprogramming (Day 30 from transduction). Antibiotic selection using puromycin was stopped 14 days into conversion.

Immunostaining

Cells were fixed using 4% PFA in PBS, pH 7.3 for 10 minutes at RT and quenched with PBS + 50 mM glycine for 5 minutes. Cells were washed 3x with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Cells were again washed 3x with PBS and treated with blocking buffer (3% BSA in PBS) for 30 minutes at RT. Staining with primary antibodies was carried out overnight in blocking buffer at 4°C. The following day secondary antibody staining was performed at RT for 45 minutes after 3x wash with PBS. Coverslips were then washed 3x with PBS and stained with DAPI (1:1000 dilution in water) for 5 mins at RT. After 3x wash with 1x PBS, coverslips were mounted using Prolong Gold (Thermo #P36930)

Western Blotting

Cells were harvested by trypsinization. Cell pellets were washed in ice-cold 1x PBS at 500 RCF for 5 minutes. Pellets were resuspended in freshly prepared RIPA buffer (50mM Tris-Hcl pH 7.4, 150mM Nacl, 1mM EDTA, 1% Triton x-100, 1% sodium deoxycholic acid, 0.1% SDS) with protease inhibitors and incubated on ice for 30 minutes. Lysates were centrifuged at 14000 RCF for 15 minutes at 4°C to remove cell debris. Supernatants were collected and assayed for total protein concentration by Bradford method. 20ug protein was prepared with 100mM Dithiothreitol and LDS sample buffer (NuPage, #NP0007), remaining lysates were aliquoted and stored at -80°C long-term. For REST detection,

lysates were loaded into 3-8% Tris-Acetate Gels (Thermo, #EA03785BOX or # EA0378BOX) and transferred to nitrocellulose membrane for 2h at 100V. Blocking was carried out using 5% non-fat dry milk in TBST (1X Tris buffered saline with 0.1% Tween) for 1h at RT. Primary antibody probing was carried out overnight at 4°C in 3% BSA in TBST, followed by incubation with appropriate secondary antibodies (either HRP-based or IRDye-based) for 2h at room temperature. REST signals were visualized either using the chemiluminescent West Pico detection kit (Thermo Fisher Scientific, catalog #34083) or LI-COR Odyssey imaging system. Western blot quantification was performed using either LI-COR Odyssey system software or Fiji image analysis with background subtraction. REST signal was normalized to loading controls recognized by anti-αβ-tubulin antibody. The following antibodies were used in all the western blotting experiments – anti-REST antibodies purified in-house in the Mandel lab by Dr. Michael Spinner – anti-REST C-terminal #720-c or #719c (1mg/mL used at 1:500 dilution), anti-REST N-terminal #p73 (1mg/mL used at 1:500 dilution), anti-αβ-tubulin (CST #2148, used at 1:1000 dilution), anti-EZH2 (CST #D2C9, used at 1:500), anti-CoREST antibody purified in the Mandel lab by Dr. Michael Spinner (1mg/mL, used at 1:500 dilution).

Overexpression of REST constructs in HEK-293 cells

Wild-type (WT) or mutated forms of human REST (K494>A or β-TRCP-MUT-WT or β-TRCP-MUT-K494>A- REST) was transiently transfected in HEK293s with Lipofectamine 2000 following manufacturer's instructions (Fisher #11668019), at 2ug plasmid per 6-well. HEK-293Ts were plated in 6-well plates (Corning) the day before transfection at ~0.5 million cells per-well. Plasmids WT-REST and K494>A-REST were obtained from the Yoo lab at WUSTL (plasmid backbone map: addgene #1436 pcDNA3 Flag HA). The mutations in the β-TRCP-binding sites in REST were made in collaboration with Dr. Sayantani Ghosh

Dastidar (Mandel lab). REST with the mutated β-TRCP-binding sites were then cloned into the addgene #1436 pcDNA3 Flag HA with or without the K494>A mutation using NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520S) as per manufacturer's instructions. Plasmids were prepared using Qiagen Plasmid DNA Mini or Midi extraction kits. Before transfection, all plasmids were sequenced and assessed for quality by agarose gel analysis.

Co-immunoprecipitation

HEK-293 cells were transfected with REST expressing plasmids (either WT-REST or K494>A-REST). Cells were harvested cells by trypsinization and washed in 1X PBS before placing pellets on ice. Cells were resuspended in ice-cold nuclei isolation buffer (50mM Tris pH 7.5, 5mM $Mgcl_2$, 25mM Kcl, 0.05mM EDTA, 0.1% NP-40, 10% glycerol and protease inhibitors) and incubated on ice for 5 mins, followed by spinning at 1000g for 3 minutes at 4°C to pellet nuclei. Pellet was then resuspended in immunoprecipitation buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% Triton X-100, 0.5mM EDTA, 10% glycerol and protease inhibitors). Samples were incubated for 30 minutes with rotation at 4°C with benzonase and spun at 15000g for 10 minutes at 4°C. Supernatants were collected and subjected to Bradford protein estimation. Lysates were then precleared using ProteinG dynabeads (Thermo, 10003D) for 4h at 4°C and washed 3x with immunoprecipitation buffer, after which 1% input was isolated and stored at -20°C. Antiboodies used for immunoprecipitation (anti-HA or anti-IgG antibodies) were conjugated to ProteinG dynabeads for 2h at RT with rotation. Immunoprecipitation of precleared lysates with anti-HA or anti-IgG-conjugated beads was carried out overnight at 4°C. Next day, beads were washed in immunoprecipitation buffer for 4 times at 4°C with rotation and resuspended in RIPA buffer with 4x sample buffer (NuPage LDS sample buffer) and DTT, and heated at

70°C for 10 minutes before subjecting them to SDS-PAGE followed by western blotting as previously described above. Input samples collected the day before were also processed for in the same manner and run alongside the immunoprecipitated samples. Quantification of resulting western blots were performed on LiCor odessey imaging system. For EZH2 quantification in Chapter 4 - Figure 4, quantity of EZH2 measured from the western blot in both WT- and K494>A- REST conditions were normalized to the corresponding amount of REST enriched in the immunoprecipitation lanes to account for differences in immunoprecipitation efficiencies between the two conditions.

Cycloheximide Chase Assay

HEK-293 cells expressing mutated or wild-type (WT) forms of REST protein were treated with cycloheximide solution (Sigma #01810 or #C4859) at a final concentration of 100ug/mL in 0.1% DMSO (Stock concentration = 100mg/mL in 100% DMSO or 355.43mM). Cells were then harvested at the appropriate timepoints by scraping in RIPA buffer (50mM Tris-Hcl pH 7.4, 150mM Nacl, 1mM EDTA, 1% Triton x-100, 1% sodium deoxycholic acid, 0.1% SDS) with protease inhibitors and incubated on ice for 15 minutes. Lysates incubated with benzonase (Millipore #E1014-25KU) at 1ul/mL and incubated with rotation for 20 minutes. Lysates were centrifuged at 14000 RCF for 15 minutes at 4°C to remove cell debris, prepared and subjected to western blotting, following by detection and quantification as described above.

Real-time quantitative PCR (RT-qPCR)

Primer design and efficiency assessment:

Forward and reverse primer pairs to amplify target sequence were designed using NCBI primer-blast. Efficiency of primer pairs was calculated using a standard curve and only

those with 90-110% efficiency used for experiments. For gene expression studies, RNA was extracted using PureLink RNA Mini kit (Thermo, #12183018A) and treated with DNAse. 1ug RNA was used for reverse transcription reactions with Super script III kit (Thermo, #18080093). qPCR was performed in an Applied biosystems Quant-studio real-time PCR instrument (Life technologies) with SYBR green PCR master-mix (Thermo, #4309155). Relative abundance of target gene was determined using a standard curve post normalization with housekeeping genes (GAPDH and 18s).

Chromatin immunoprecipitation (ChIP)

Cells (Human fibroblasts or iNs) harvested by trypsinization were washed with ice-cold 1x PBS and pelleted (5 mins, 500 RCF, 4°C). Pellet was resuspended in cross-linking buffer containing 1% formaldehyde (10mM HEPES, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1M sodium butyrate, fresh protease inhibitors) and incubated at RT on shaker for 10 mins (650ul for 4 million cells). Fixation was quenched with 2.5M Glycine in PBS for 2mins at RT on a shaker and cells were pelleted by centrifugation (5 mins, 500 RCF, 4°C). Cells were then washed with ice-cold 1x PBS and pelleted (5mins, 500 RCF, 4°C). Pellet was either stored at -80°C or processed right away for nuclei isolation followed by ChIP.

Nuclei isolation:

Cell pellet (either fresh or frozen) was resuspended in HB buffer (250mM sucrose, 25mM KCl, 5mM $MgCl_2$, 20mM Tricine-KOH pH 7.8, 1M DTT, 0.15M spermine, 0.5M spermidine, 1M sodium butyrate, fresh protease inhibitors) on ice (1mL for 4 million cells). Cells were then homogenized with dounce homogenizer (5 strokes each with loose (A) and tight (B) pestle). 0.3% NP-40 was added followed by homogenization with tight pestle (B) for 5 more times. Cells were spun at 4000 RCF at 4°C for 5 mins. Nuclei was washed in 1mL

L3 buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 1mM EGTA), spun at 4000 RCF for 5 mins at 4°C and resuspended in 500uL L3 buffer. 5uL was isolated for counting on hemocytometer and DAPI staining after 1:20 dilution with L3 buffer. Nuclei was stored at 4°C if sonicating the next day or -20°C for long term.

Chromatin Fragmentation:

15uL of 10% SDS was added to the nuclei in L3 buffer and gently mixed by hand. Chromatin shearing was performed on Covaris S220 ultrasonicator 12 minutes (200 cycles per burst, 5% duty cycle and power level at 4 MPSSR core) and 5uL each of sonicated and un-sonicated sample was placed at 65°C overnight in hybridization oven to reverse crosslink followed by agarose gel analysis for verification of fragmentation. Protein concentration in sheared chromatin was estimated using bradford protein assay (Bio-rad). Glycerol (20%) was added to the sheared nuclei and stored at -20°C until further use.

Immunoprecipitation

Sheared chromatin was spun at 16000 RCF for 5 mins at 4°C to remove any impurities and made up to a volume of 1000uL with L3 buffer and 3X Covaris ChIP buffer (20mM Tris-HCL pH 8.0, 3% Triton X-100, 450mM Nacl, 3mM EDTA). Dynabeads Protein G beads (Thermo, 10003D, 60uL per reaction) were washed 3x with TBSTB (1x TBS, 1% Triton-X 100, 1% BSA, fresh protease inhibitors) at RT for 5 min each and resuspended in 300uL TBSTB. Sheared chromatin was pre-cleared with 100uL of equilibrated Dynabeads for 4h at 4°C with end-to-end rotation. After preclearing 5% of ChIP input was taken and stored at -20°C. For antibody immobilization, 200uL of equilibrated Dynabeads were incubated with 20ug REST-C antibody (#719, Mandel lab) or 40ug H4K20Ac (gift from Kimura lab, Tokyo institute of Technology) or 20ug EZH2 antibody (CST, D2C9) or

20ug CoREST antibody (#p71, Mandel lab) with end-to-end rotation at RT for 2 hours, after which the beads were washed 3x with 1mL TBSTB at RT. Chromatin immunoprecipitation was performed overnight at 4°C with end-to-end rotation after adding the antibody immobilized beads to the precleared chromatin lysate. The next day, beads were isolated and washed sequentially twice with low-salt wash buffer (0.1% SDS, 1% Triton-X100, 20mM Tris-Hcl pH 8.0, 150mM NaCl, 2mM EDTA), high-salt wash buffer (0.1% SDS, 1% Triton-X100, 20mM Tris-Hcl pH 8.0, 500mM NaCl, 2mM EDTA) and LiCl wash buffer (250mM LiCl, 1% NP-40, 1mM EDTA, 10mM Tris-Hcl pH 8.0, 1% NaDeoxycholate) for 5 mins each at 4°C with end-to-end rotation. Beads were washed twice with 1mL TE buffer (10mM Tris-Hcl pH 8.0, 1mM EDTA) at RT for 5 mins. Beads and input samples were then directly mixed with 200uL and 150uL, respectively, of elution buffer (10mM Tris-Hcl pH 8.0, 300mM NaCl, 5mM EDTA, 0.5% SDS), and incubated at 65°C in hybridization oven to reverse cross-link DNA overnight. The next day, 10ug of RNAase was added to each sample and incubated for 30 mins at 37°C followed by incubation with proteinase K for 1-2 hours at 55°C. DNA was then extracted with 25:24:1 Phenol/Chroform/isoamyl alcohol and isolated using QIAGEN PCR purification kit with two elutions of 30uL (total of 60uL).

ChIP-qPCR:

ChIP was validated by qPCR. Forward and reverse primer pairs to amplify genomic regions were designed using NCBI primer-blast. Human genomic DNA (isolated from LUHMES or HEK-293Ts) was used for standard curve. Percent input analysis was performed to quantify the amount of DNA pulled down with the antibody relative to the amount of starting material (input).

REST ChIP-Seq and analysis

This was performed with technical inputs from Dr. Andrew Adey at OHSU. ChIP-DNA was provided to the Massively Parallel Sequencing Shared Resource (MPSSR) core at OHSU for library generation followed by deep-sequencing. Libraries were profiled and concentrations estimated using TapeStation D1000 DNA tape. Sequencing was performed on the NovaSeq by MPSSR and the resulting fastq files were provided for analysis. The quality of sequenced reads were verified using FastQC. Reads were then mapped to the reference genome (hg38) using HiSat2. Duplicate reads were removed using Picard, following which extraction of genomic regions enriched in the ChIP samples, compared to their corresponding input samples, was performed using MAC2 peak caller. In order to identify peaks containing canonical REST motifs (Full RE1 and left or right RE1 half-sites), as previously published (McGann et al., 2021), motif analysis was performed using MemeSuite tools. Lastly, gene annotation for ChiP-Seq peaks were performed using ChiPSeeker (R Bioconductor) and reactome pathway analysis using Panther database.

Chapter 3: Limitations of an *in vitro* human neuronal aging system revealed by studying REST

Abstract

For neurons to function throughout their long lifetime they must maintain their unique gene expression profiles. Despite advances in invertebrate and rodent models, the underlying mechanisms for preserving gene expression in the human brain are not known. This is in part due to the evolution of human specific gene expression patterns. Consistent with this idea, REST was shown recently in our lab, unexpectedly, to have an expression pattern in the aging human brain opposite to that of the mouse brain, pointing to human-specific functions for REST during aging. Specifically, in mouse, REST levels are nearly absent in the aging adult brain, while in the human brain, REST levels are low in young ages, become elevated at later ages, and remain elevated even in nonagenarians. Moreover, the REST target genes identified in the aged human hippocampus represent novel non-canonical genes, distinct from the majority of the canonical genes bound by REST in the mouse hippocampus. Furthermore, previous studies by other groups have proposed neuroprotective roles for REST during brain aging, which while intriguing have also been controversial. Lastly, given that these studies were conducted in archived human brain tissue, the mechanisms of REST function during healthy brain aging remains unknown. Recent advances in the field of direct cellular reprogramming have given rise to new strategies that allow for *in vitro* generation of human neurons that model aging. These approaches involve direct lineage conversion of terminally differentiated human somatic cells into neurons, which does not involve any embryonic cellular states, thereby preserving age-associated molecular signatures that are typically lost during the differentiation process. One such popular approach is the use of brain-enriched microRNAs (miR) to forcibly convert human dermal fibroblasts to neurons, also known as induced neurons (iNs), which recapitulate cellular and molecular features of aging. Most importantly, miR-derived human iNs have been reported to preserve an epigenome-

related aging feature, suggesting their utility in studying epigenetic aging factors, such as REST. Here, in my study, I utilized miR-derived iNs from aged human donors to gain insights into REST function during brain aging. However, upon characterizing REST in aged human iNs, I, unexpectedly identified major limitations in this model that precluded me from studying REST function. My findings question the extent to which iNs truly represent neurons of the aging human brain and maybe useful for other investigators interested in using iNs to study aging associated transcription factors.

Introduction

The longevity of humans poses a unique challenge to neurons, which as post-mitotic cells, must survive and perform their functions throughout our entire lifetime. Precise regulation of gene expression plays a critical role in maintaining neuronal function for a healthy brain. Accumulating evidence indicates very little overlap in gene expression patterns between the mouse and human brain during aging (Zahn et al., 2007; Loerch et al., 2008; Swindell et al., 2012; Yang et al., 2015), suggesting that certain mechanisms required for maintaining a healthy brain are fundamentally different in these species. Consistent with this idea, members of the Mandel lab identified completely opposing age-dependent expression patterns of the unique neuronal transcription factor, REST, in mouse and human brain. In mice, REST levels are highest in early adulthood and decline with age, whereas in humans REST levels are low during early adulthood, increase by the end of the third decade of life and remain elevated even in nonagenarians. Moreover, their study also identified a new set of genes related to innate immunity bound by REST, unique to the human hippocampus, suggesting a mechanistic role for REST in the aging human brain (McGann et al., 2021).

This exciting discovery was made in archived human brain tissue, but, to study REST-mediated mechanisms, optimally we needed an experimental system that recapitulated aging features in brain cells, allowed for manipulation of REST levels, and was amenable to large scale molecular and biochemical assays. In recent years, development in the neuronal reprogramming field has enabled direct conversion of easily accessible cells such as human dermal fibroblasts, to neurons using varied approaches including microRNAs, transcription factors, small molecules or a combination of such factors (Yang et al., 2011). This trans-differentiation approach has been highlighted as more suitable for retaining certain age-associated features (Mertens et al., 2015; Huh et

al., 2016; Tang et al., 2017), in particular the epigenetic clock (Huh et al., 2016) , which is erased in alternate approaches involving the generation of intermediate embryonic-like pluripotent cells. Epigenetic clock is a molecular estimator of biological age, used to predict the age of cells/tissues by assessing the methylation status of 353 CpG sites in the genome (i.e. methylation of a cytosine nucleotide which is followed by a guanine nucleotide separated by a phosphate group in the linear sequence of bases). The discovery of the 353 CpG sites came from applying a supervised machine learning algorithm on DNA methylation data collected from 51 human tissues/cell-types (Horvath, 2013). Age-correlative methylation changes in the 353 CpG sites (either hypo- or hyper-methylation) were identified to be sufficient to predict biological age of cells/tissues. Moreover, a comparative review of six molecular age estimators, namely the epigenetic clock, telomere length, transcriptomic-based, proteomic-based, metabolomic-based and composite biomarkers-based methods concluded the epigenetic clock to be the most promising age predictor (Jylhävä et al., 2017). Lastly, this epigenetic clock method was employed by Huh and colleagues, to demonstrate for the first time, that human iNs derived using microRNA-9/9* and microRNA-124 (miR-9/9*-124) from adult donor dermal fibroblasts retain their corresponding epigenetic ages (Huh et al., 2016). In other words, the predicted biological age of iNs derived from an 80y old or a 20yr old donor's fibroblasts using miR-9/9*-124, based on their DNA methylation status at the 353 CpG sites, were 80y and 20y, respectively.

Based on the findings showing the preservation of the epigenetic clock in human iNs (Huh et al., 2016), and studies from the Mandel lab proposing new roles for elevated REST in the aging human brain (McGann et al., 2021), I hypothesized that REST would regulate a novel set of genes in the aged human iNs, distinct from that of young iNs. In order to test this hypothesis, I first generated iNs using miR-9/9*-124 from aged human donor fibroblasts and characterized REST expression and chromatin binding profiles to

identify REST target genes. Unexpectedly, I uncovered that this miRNAs-mediated reprogramming strategy likely generated immature iNs, in which REST still remained bound to its canonical neuronal genes, similar to that of the parental fibroblasts. I also identified great inter-individual variability of REST profiles in aged human donor fibroblasts. Together, the data precluded my ability to gain insights into a role for REST in human brain aging, and more generally highlights major limitations in such *in vitro* aging systems depending on the phenotypes being interrogated.

Results

Validation of canonical role for REST in aged human fibroblasts

In order to first confirm REST expression in human dermal fibroblasts, I performed immunostaining and western blotting for REST in cells from a healthy 68-year old donor obtained from the Coriell Institute, using anti-REST antibodies generated in our lab. The health records for this donor at Coriell states the individual was healthy, with no diagnosis of any active disease, at the time of sample collection. Figure 1A shows predominantly nuclear expression of REST in the fibroblasts as expected. REST migrated at its expected size (~200 kDa) in SDS-PAGE visualized in a western blot (Figure 1B). To validate the canonical role for REST, where it binds to a large 21-bp RE1 motif in neuronal genes to repress their expression (as described in Chapters 1.1 – 1.2), I performed quantitative chromatin immunoprecipitation (qChIP) on two well-studied neuronal genes regulated by REST, namely, the glycine receptor alpha 1, GLRA1 and RNA Binding Fox-1 Homolog 3, Rbfox3. Figure 1C shows enriched REST binding to the RE1 sites in the GLRA1 and Rbfox3 genes, in comparison to its binding at a site within the coding sequence (CDS) of the two genes used as negative control for the qChIP. Since REST is also known to recruit co-repressor proteins, such as CoREST, to its target sites in neuronal genes, I performed

a qChIP for CoREST binding at the same sites as in Figure 1C, and observed an enriched binding of CoREST at the RE1 sites within the GLRA1 and Rbfox3 genes, as expected (Figure 1D). Interestingly, I also detected an enrichment of H4K20Ac, a unique repressor histone mark recently discovered to be associated with REST in human HeLa cells, preferentially at the RE1 site in GLRA1 gene and not in the Rbfox3 gene (Figure 1E). This result is similar to a previous observation in the human hippocampus (Figure 1F, credit: Dr. Saurabh Garg, Mandel lab alumni), suggesting H4K20Ac is only associated with a subset of REST regulated genes in human fibroblasts, similar to that of the human brain. Together, these results confirm a canonical role for REST in human dermal fibroblasts from a 68y old healthy donor.

Generation of aged human iNs from dermal fibroblasts using miRNAs

Having established REST expression and canonical binding to neuronal genes in the 68-year old donor dermal fibroblasts, I induced neuronal differentiation in these cells using miR-9/9* and miR-124 (miR-9/9*-124) as previously published (Richner et al., 2015). Figure 2A, illustrates an adaptation of the key steps to generate these iNs, which took several weeks of optimization. At the end, I confirmed neuronal identity at 30 days post transduction with miR-9/9*-124 by validating the expression of pan-neuronal markers such as NeuN, MAP2 and Tuj1 (Figure 2C) and showed upregulated mRNA levels of neuronal genes such as Synapsin1 (55 fold) and Tuj1 (4 fold), similar to previous publications (Figure 2B). Nearly all differentiated cells (~98%) by Day 30 stained positive for NeuN (determined by manual counting), indicating a homogenous population of human iNs.

miR-9/9*-124-derived human iNs exhibit REST binding to neuronal genes and are likely immature

After optimizing culture conditions for large-scale long-term (30 days) generation of aged human iNs, I next wanted to characterize REST expression in these cells. I performed immunostaining, western blotting and qChIP chromatin binding analysis of REST in iNs differentiated from the 68y old healthy donor fibroblasts. Figure 3A shows a predominantly nuclear expression of REST in the aged human iNs, co-stained with a neuronal marker, MAP2 (Figure 3A). However, I was unable to detect REST in the human iNs by western blotting, as shown in Figure 3B, suggesting REST levels are low in the iNs compared to that of the parental donor fibroblasts. Surprisingly, by qChIP, I was still able to detect enriched REST binding to the RE1 sites in both the GLRA1 and Rbfox3 genes, as they were in the parental fibroblasts (Figure 3C). Even more surprising, was the enrichment of CoREST at the RE1 sites in the GLRA1 and Rbfox3 genes (Figure 3D), also similar to that of the donor fibroblasts, suggesting not only the presence of REST at these sites, but likely also other components of its repressor complex. Of note, the fold enrichment of REST binding, i.e., the amount of increase in REST binding at RE1 site compared to the CDS (negative control site) in both GLRA1 and Rbfox3 genes, was lower in the iNs compared to that of the donor fibroblasts (Figure 3E). This likely reflects the drop in REST levels in the reprogrammed iNs, as evidenced by the western blot detection of REST levels in Figure 3B.

Comparison of REST ChIP-Seq in human iNs and parental fibroblasts reveals direct reprogramming by miR-9/9*-124 does not remove REST from neuronal gene chromatin

My previous REST qChIP experiments showed an overlap in REST target genes between the aged iNs and parental fibroblasts. However, based on their distinct cellular

identities, I expected to find some non-overlapping cell-type specific REST targets for further study. To test this hypothesis, I performed a REST ChIP-Seq experiment in both the iNs and the parental fibroblasts and analyzed the sequencing data as detailed in the methods section (Refer to ChIP-Seq analysis in Chapter 2), with Dr. Andrew Adey's inputs, which were very helpful in this regard.

The total number of REST peaks (i.e. DNA regions enriched for REST binding) were lower in the iNs compared to the donor fibroblasts (856 vs 1378, Figure 4A), with a majority of peaks (73% or 625 peaks, Figure 4A) identified at the same genomic location as in the fibroblasts. The remaining 27% (or 231 peaks, Figure 4A), were uniquely bound in the iNs, but upon closer inspection were very far away (>20kb) from the nearest transcription start site (TSS), or likely located at false peaks at repetitive sites such as peri-centromeric regions.

Next, I sought to determine whether the peaks identified by ChIP-Seq contained RE1 sequences, the canonical binding motif for REST. To do this, I performed motif analysis using Meme-ChIP suite of tools (Machanick & Bailey, 2011), where I defined RE1 sites as either a consensus full, left or right half site (Figure 4D), as previously described (McGann et al., 2021). As expected, the majority of total peaks in both cell types contained RE1 motifs (59% and 58% of total sites in fibroblasts and iNs, respectively, Figure 4B). The remaining 41% and 42% of the total peaks in the fibroblasts and iNs, respectively, consisted of non-RE1 sequences. Upon detailed examination of these non-RE1 containing peaks, I observed no biologically relevant evidence of REST recruitment to these sites. First, most of them were too far away from the nearest TSS (>20kb). Second, I did not find any other transcription factor binding motifs at these non-RE1 sequences that would suggest an indirect recruitment of REST to these loci. The remainder of my analysis was thus performed only on the peaks containing RE1 (either full or half) sites, as illustrated in Figure 4D.

Nearly all RE1 containing peaks (97% or 481 peaks, Figure 4C) in iNs overlapped with that of the parental fibroblasts, indicating REST is not entirely removed from neuronal gene chromatin during reprogramming, as previously claimed (Lee et al., 2018). Figure 4E shows ChIP-Seq tracks representing REST peaks at the RE1 sites associated with six neuronal genes, namely βIII tubulin, Synapsin1 (Syn1), glycine receptor alpha 1 (GLRA1), serotonin receptor 5A (5-hydroxytryptamine or HTR5A), neuronal PAS domain protein 4 gene (NPAS4), and Synaptotagmin 5 (Syt5), in both iNs and parental fibroblasts. Figure 4E also shows that the REST RE1 peaks in the iNs have smaller peak heights compared to that of the fibroblasts, which is in accordance with my observations of a decreased fold enrichment of REST binding at the RE1 sites of its neuronal genes as shown in Figure 3E, likely due to the low levels of REST in the iNs compared to the parental fibroblasts (as shown in Figure 3B). As expected, based on the large overlap in REST targets between the iNs and fibroblasts, the reactome enrichment categories were similar between the two cell types, as shown in Figure 4F (fibroblasts) and 4G (iNs). Although there were differences in the order of significance of the enrichment pathways between the two cell types, the top category in both cells was the neuronal system, representing canonical REST target genes such as SCN10a (sodium voltage gated channel), KCNQ2 (potassium channel), CACNA1A (calcium channel), BDNF (neurotrophin), SNAP-25 (presynaptic gene) etc,.

Inter-individual variability of REST in aged human fibroblasts

Before deciding whether to repeat the REST ChIP-seq analysis for biological replicates, I characterized REST expression and chromatin binding profiles in dermal fibroblasts from multiple donors. Table 5A shows donor details of the four age-matched dermal fibroblasts I used in this experiment (IDs 1-4). I also used fibroblasts from a young donor (22y, ID 5 in Table 5A). Figure 5B and 5C show REST protein detection in all the

donor fibroblasts using a C-terminal and an N-terminal targeted antibody against REST, respectively. The total levels of REST were highly variable (2-3 fold) between the age-matched fibroblasts (IDs 1-4) as shown in the corresponding quantification plots in Figure 5B and 5C. Most importantly, two out of the four fibroblast-lines from the aged donors (Donor IDs 3 and 4), had similar levels of total REST as that of fibroblasts from a young individual (Donor ID 5), suggesting no age-correlation. Apart from total REST levels, the amount of REST bound to chromatin was also highly variable in the different fibroblasts' lines. Figure 5D shows qChIP for REST binding at the RE1 and CDS sites of the GLRA1 gene for the four fibroblast lines from age-matched healthy donors (Donor IDs 1-4, Refer Table 5A). The fold enrichment of REST binding at the RE1 site was highly variable between all four individuals. Even the two fibroblast lines which had the highest levels of total REST protein by western blotting (Donor IDs 1 & 2), had very high variability in REST binding to its target gene, with an 84-fold and 1-fold enriched binding at the RE1 site in GLRA1 gene, respectively. Collectively, these results prevented me from using these lines for any further experiments related to studying REST function.

Discussion

In this study, I generated iNs from aged human fibroblasts using miR-9/9* and miR-124 and identified limitations associated with this system to study REST in the context of neuronal aging. Although the aged iNs exhibit some pan-neuronal features based on morphology and expression of neuronal marker genes, which are distinct from the fibroblasts, they exhibited identical genome-wide REST binding profiles to that of their parental fibroblasts, leading me to conclude that the "neurons" were not very differentiated and that reprogramming was incomplete. I also identified great variability in total REST protein levels and REST binding to target gene chromatin among the purchased human

fibroblasts derived from donors of the same age, further diminishing my confidence in utilizing this system to study REST mechanisms in the context of aging.

As a transcriptional repressor of neuronal genes in non-neuronal cells, REST binds to the well-characterized RE1 DNA motif and facilitates neuronal gene repression through the recruitment of co-repressors such as CoREST, and histone deacetylases that are associated with gene repression. In agreement with this function, REST is predominantly nuclear in human fibroblasts from a 68y old donor (Figure 1A), binds to canonical RE1 sequences associated with neuronal genes, such as the glycine receptor gene GLRA1 and RNA-binding protein fox1 gene, Rbfox3 (Figure 1C), and recruits CoREST to their RE1 sites (Figure 1D) for target gene repression. The chromatin-remodeling mediated by REST for neuronal gene repression is dynamic and complex, consisting of well-studied histone modifications, such as, deacetylation, but also novel modifications whose mechanisms are yet to be elucidated. One such novel histone mark, recently discovered to be associated with REST binding in human cells, whose mechanism is not fully understood, is the acetylation of histone H4 at lysine 20 (H4K20Ac) (Kaimori et al., 2016). Part of its novelty is that histone acetylation is usually associated with gene activation, not repression, and H4K20Ac levels have been shown to decrease in aged archived human brain tissues (Nativio et al., 2020). Interestingly, H4K20Ac is preferentially associated with the RE1 site of the Rbfox3 gene in the 68y old dermal fibroblasts (Figure 1E), but not with other RE1-containing genes. Moreover, a previous study performed in the Mandel lab also showed preferential association of H4K20Ac with REST at the Rbfox3 gene in aged in-vivo human hippocampus (68y) (Figure 1F). However, since the enzyme responsible for depositing the H4K20Ac mark in human cells is currently unknown, the molecular association between REST and H4K20Ac, and the reason for its preferential association at certain genomic sites remains an open question that should be explored in future studies.

Ever since human iNs were generated from dermal fibroblasts for the first time in 2011 (Ambasudhan et al., 2011), the field of direct neuronal reprogramming has progressed rapidly. Currently, there are varied approaches to generate human iNs, including the use of transcription factors, microRNAs, pharmacological agents, or a combination of such factors. Amongst all these methods, the approach using miRs-9/9* and -124 reported the highest conversion efficiencies, particularly from dermal fibroblasts of aged human donors, as opposed to other methods which predominantly used fetal human fibroblasts (Richner et al., 2015; Yoo et al., 2011). I adapted this miR-based strategy for long-term (30 days), large-scale generation of human iNs from aged donors and was able to achieve nearly, 98-100% of the iNs expressing pan-neuronal markers such as NeuN (Figure 2A), indicating successful high-efficiency conversion of fibroblasts to a homogenous population of iNs, as previously described. I also confirmed upregulated expression of pan-neuronal gene transcripts, such as Synapsin1 and Tuj1 (or βIII tubulin), in aged iNs compared to the donor fibroblasts (Figure 2B), also consistent with previous published findings (Abernathy et al., 2017). However, despite these distinct pan-neuronal features, REST was still bound at the RE1 sites of neuronal genes (Figure 3C and Figure 4), albeit at lower levels compared to that of the donor fibroblasts, indicating that less of the neuronal gene chromatin is bound by REST in the iNs compared to the fibroblasts. These findings highlight three aspects – 1) The overall levels of REST are low in the human iNs compared to the fibroblasts, as predicted by reduction of REST during neuronal differentiation from previous studies, 2) human iNs are likely immature, or in between the cellular states of a progenitor and a mature neuron, similar to that of *in vitro* bona fide neural progenitors where REST cannot be detected by western blotting but remains bound to its neuronal gene targets by qChIP (Ballas et al., 2005), and 3) The upregulated expression of REST target genes, such as Synapsin1 and Tuj1 (or βIII tubulin) in the iNs

(Figure 2B) is due to the reduced binding of REST at their RE1 sites, as evidenced by the lower peak heights of REST binding in the ChIP-Seq analysis (Figure 4E).

Lastly, from prior studies we know miR-9/9* can downregulate REST and miR-124 and REST share a reciprocal relationship in the establishment of neuronal identity (Conaco et al., 2006), wherein REST removal from its binding to the RE1 site associated with the miR-124 gene causes up regulation of the miRNA, which in turn downregulates non-neuronal genes, in differentiated neurons (Refer Chapter 1.4.1.1 for detailed background). Previous studies that analyzed global transcriptome changes during the miRNAs-mediated reprogramming of human fibroblasts to iNs reported no change in REST mRNA levels (Abernathy et al., 2017), suggesting that miR-9/9* and miR-124 is not regulating REST during the reprogramming process.

Finally, human iNs-based studies are expensive, laborious and often involve a limited number of biological replicates (typically 3-10 lines). In order to detect the desired experimental effect of a biological factor, such as REST, with high statistical power, the variability associated with the factor will have to be minimal across multiple biological samples. Unfortunately, REST profiles were highly variable in the age-matched donor fibroblasts themselves (Figure 5), making it difficult to compare any REST ChIP-Seq experiments or having to profile REST in fibroblast samples from a large number of donors, to see whether variability would be decreased. This variability could be attributed to the heterogenous nature of the primary donor fibroblasts, which are cultured and expanded from human skin biopsies. Human fibroblasts can vary in their properties depending on the location of the biopsies, the genetic background of the individual and differences in their exposure to the outside environment. Moreover, the human skin is the primary barrier of defense against environmental stressors, which can influence their cellular characteristics, independent of the individual's age. While most studies have assessed transcriptional variability between donors in human based *in vitro* systems,

variability assessment at the level of the proteome is just getting started. For example, proteome variability was assessed recently in a human *in vitro* model of astrocytes using mass spectrometry, wherein the largest source of variability was from inter-individual differences (Beekhuis-Hoekstra et al., 2021).

In retrospect, while iNs offer distinct advantages over human post-mortem brain tissues for mechanistic studies, their disadvantage is the lack of a standardized criteria used to define their similarity to that of the aging mature neurons of the human brain. Current methods used to assess maturity of human iNs are based on the expression of a few pan-neuronal markers and the preservation of the epigenetic clock, both of which might be too narrow of a criterion to determine whether human iNs represent bona-fide neurons of the in-vivo aging human brain.

Figures and figure legends

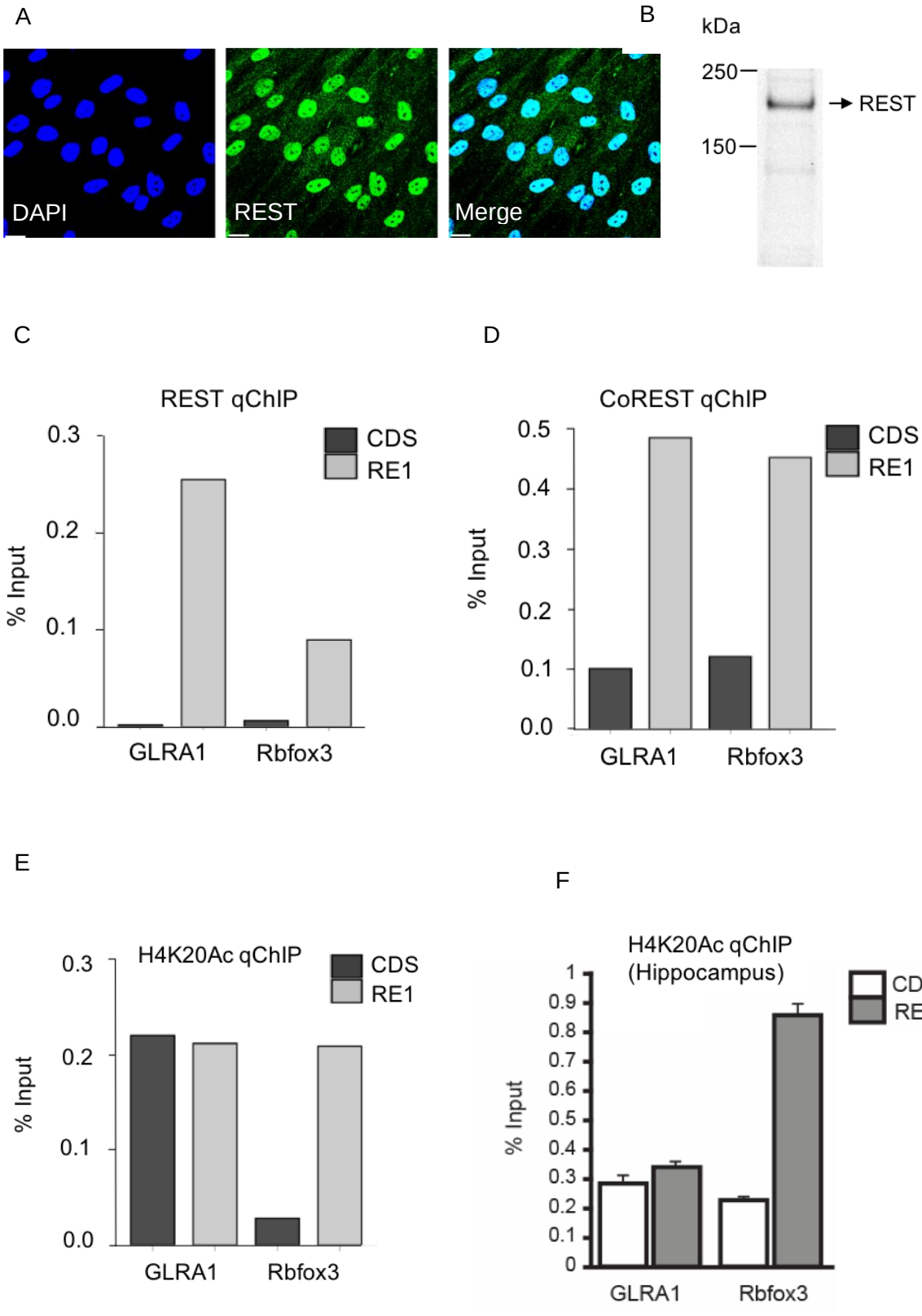


Figure 1. Characterization of REST in human primary fibroblasts from a 68y old donor (AG04349A, Coriell) A) Representative images of human fibroblasts immuno-stained for REST and DAPI (nuclear stain). Scale = 20 μm. B) Western blot showing REST detection at ~200 kDa in whole cellular lysates from human fibroblasts. The sizes on the left are from protein molecular weight markers run alongside the lysates in the western blot experiment. C-F) qChIP analysis showing C) enriched REST binding at RE1 sites in genes glycine receptor alpha 1 (GLRA1) and RNA-binding protein fox1 homolog 3 (Rbfox3), D) enriched Co-REST binding at RE1 sites in GLRA1 and Rbfox3 genes, E) & F) preferential H4K20Ac enriched binding at RE1 site in Rbfox3 gene using anti-H4K20Ac antibody (Gift from the Kimura Lab, Tokyo Institute of Technology) in (E) human fibroblasts and F) human hippocampus (credit : Saurabh Garg, Mandel lab alumni). Error bars in Fig F) represent standard deviation from n=2 biological replicates. Data presented from A)-E) is from a single 68y old donor (AG04349A, Coriell institute). For C-F) Primers for a region within the coding sequence of the two genes (CDS) served as negative control for REST or CoREST or H4K20Ac antibody pulldown.

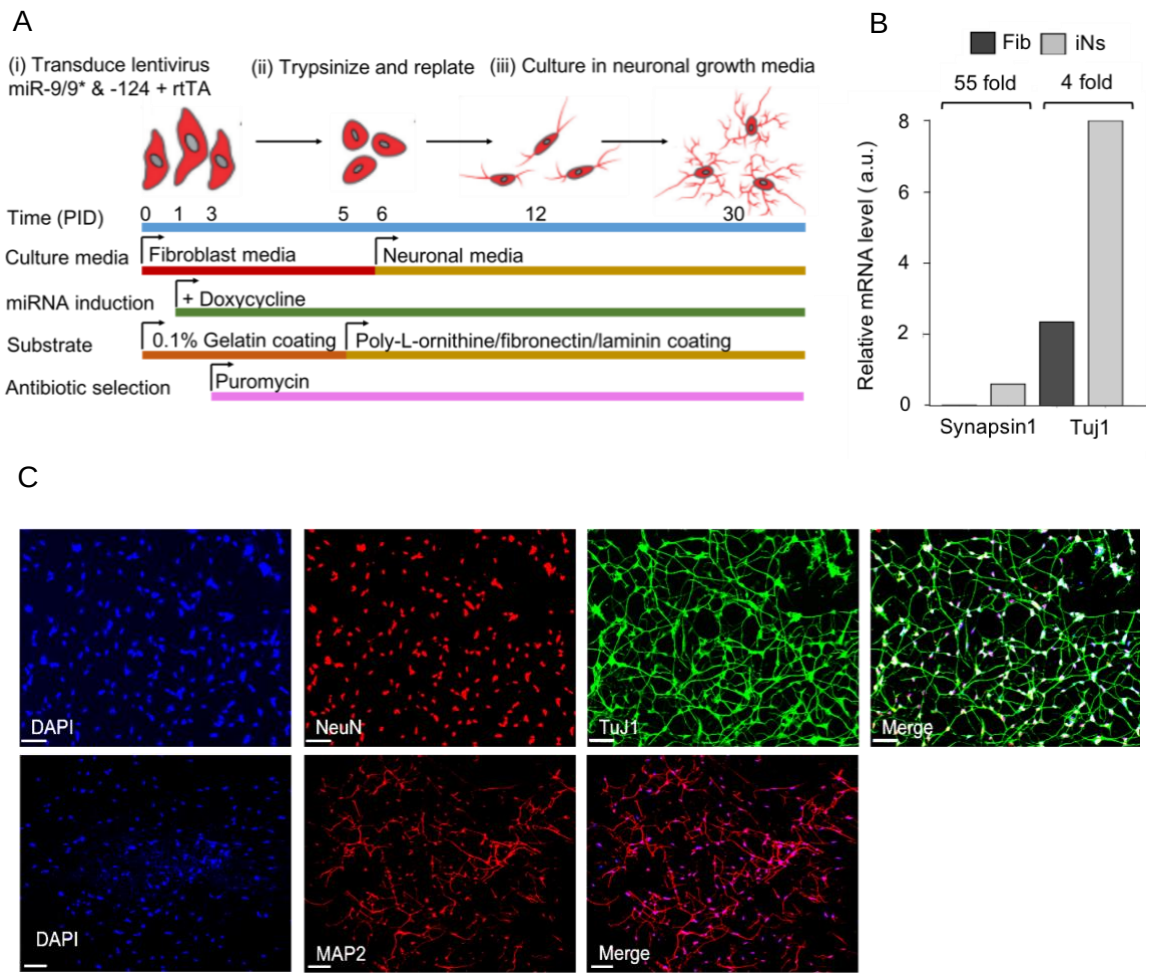


Figure 2. Direct reprogramming of human fibroblasts to iNs using miRNAs

A) Schematic of miRNAs-based reprogramming protocol adapted from Richner et al., 2014. B) RT-qPCR analysis showing upregulated mRNA levels of neuronal genes, Synapsin1 and Tuj1, normalized to 18s and GAPDH, in iNs compared to parental fibroblasts (Fib), C) Representative images showing Day 30 human iNs immuno-stained with antibodies against neuronal marker genes NeuN, MAP2 and Tuj1, Scale bar = 100 μm. Data presented here is from a single 68y old donor (AG04349A, Coriell institute).

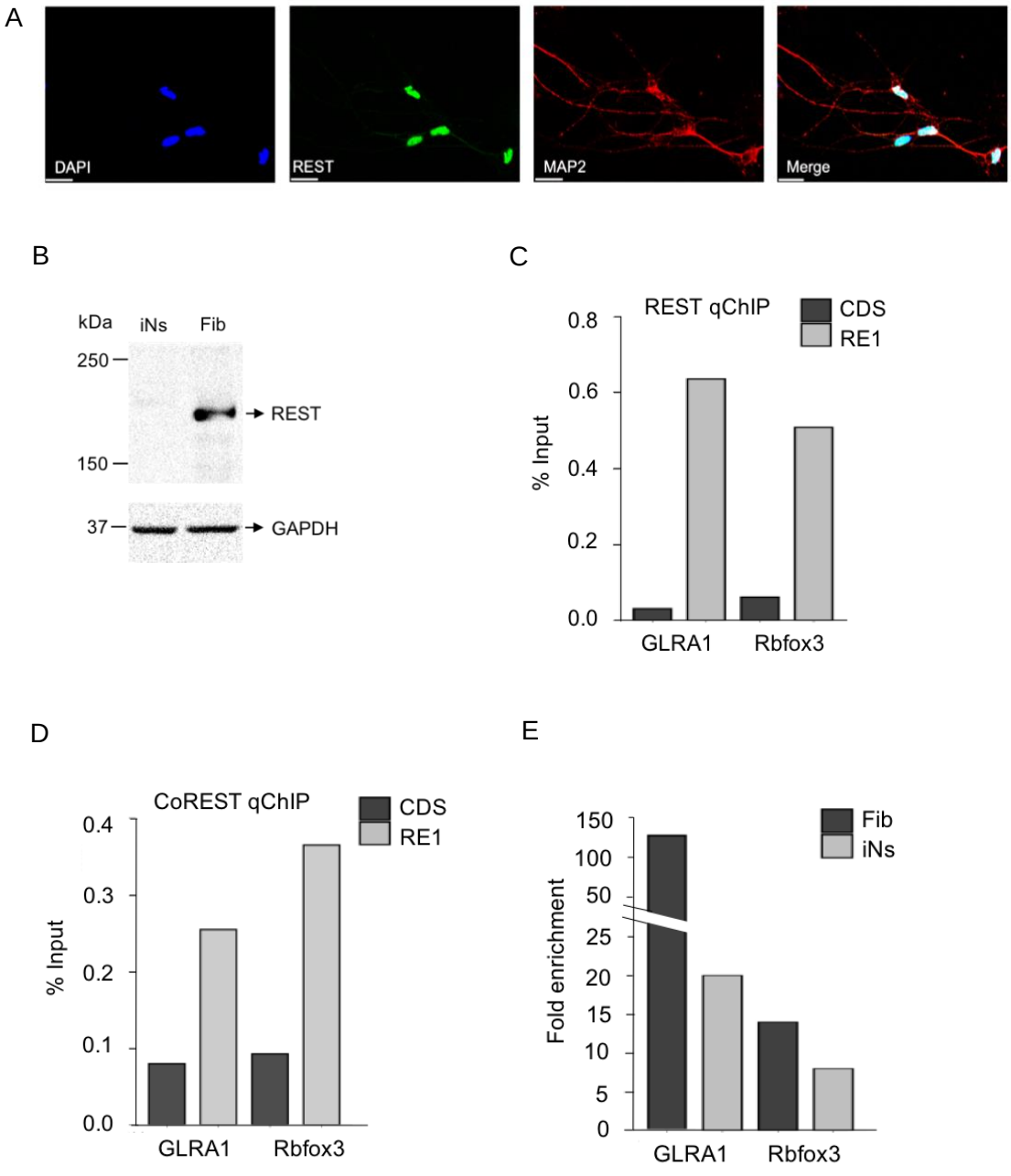
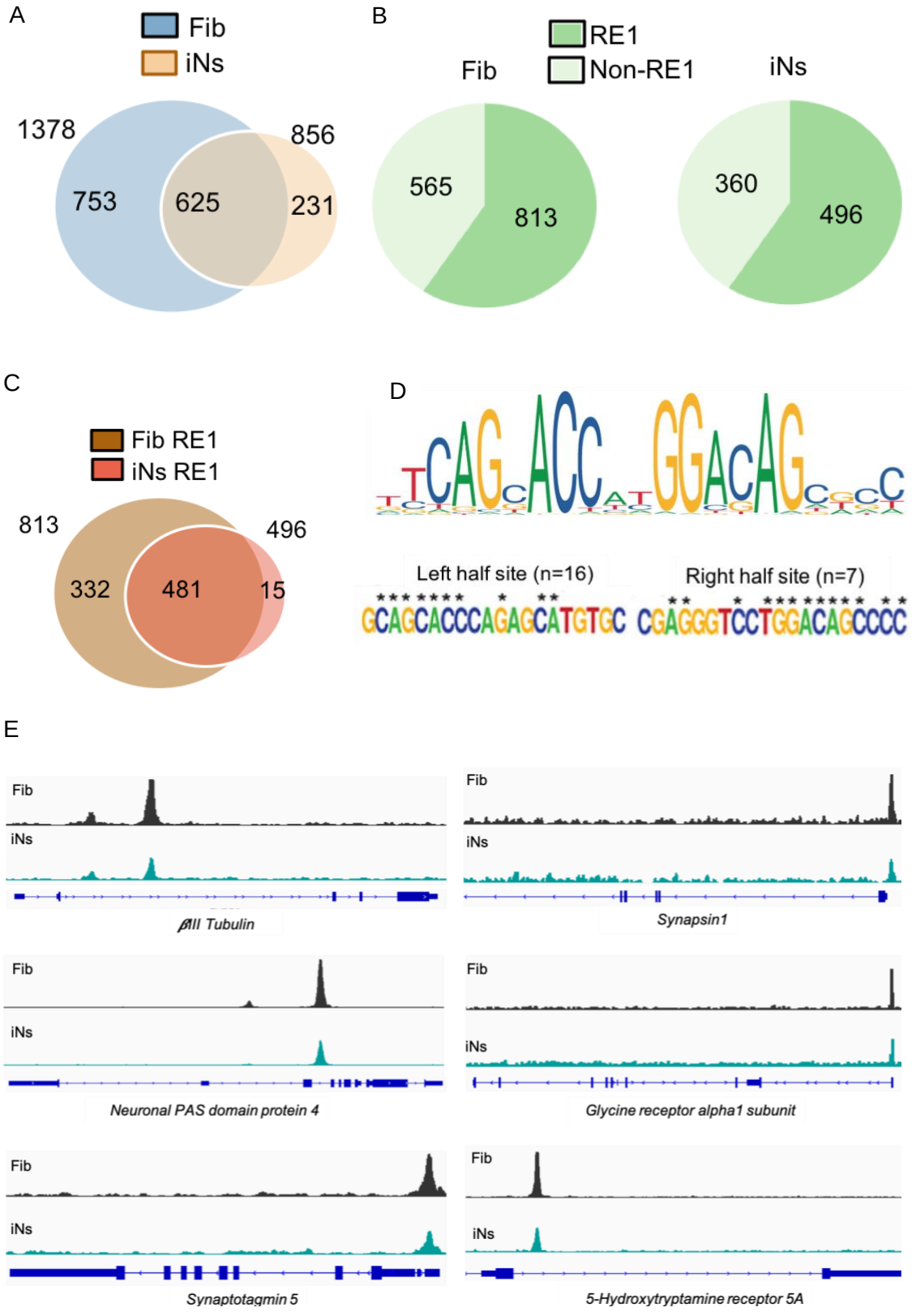
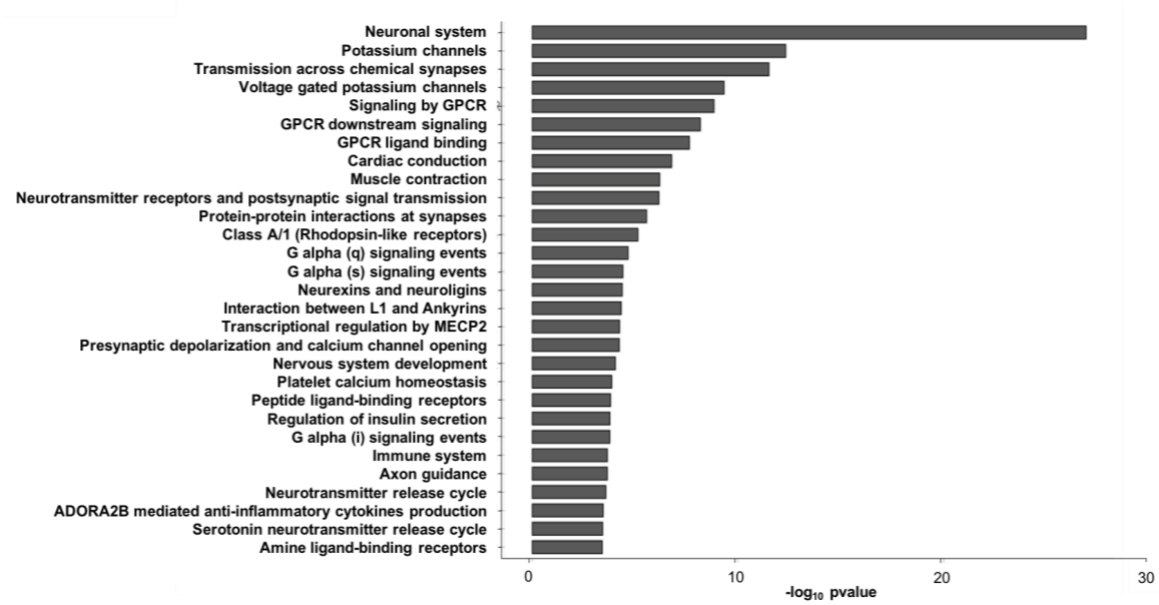


Figure 3. Characterization of REST in aged human iNs from a 68y old donor (AG04349A, Coriell Institute). A) Representative images of Day 30 human iNs immunostained for REST, neuronal marker MAP2, and DAPI nuclear stain. B) Western blotting showing REST detection at ~200 kDa in donor fibroblasts but not in human iNs in whole cellular lysates. The sizes on the left are from protein molecular weight markers run alongside the

lysates in the western blot experiment. C-D) qChIP analysis showing C) enriched REST binding at RE1 sites in glycine receptor alpha 1 (GLRA1) and RNA-binding protein fox1 homolog 3 (Rbfox3) genes, D) enriched Co-REST binding at RE1 sites in GLRA1 and Rbfox3 genes, E) Bar-plot comparison of fold enrichment of REST binding at RE1 site relative to a control site (CDS) within GLRA1 and Rbfox3 genes in parental fibroblasts (Fib) vs iNs. For C-D) Primers for a region within the coding sequence of the two genes (CDS) served as negative controls for REST or CoREST antibody pulldown.


A
Fib
iNs
1378
856
753
625
231
B
RE1
Non-RE1
Fib
iNs
565
813
360
496
C
Fib RE1
iNs RE1
813
496
332
481
15
D
Left half site (n=16)
Right half site (n=7)
GCAGCACCCAGAGCATGTGC
CGAGGGTCCTGGACAGCCCC
E
Fib
iNs
βIII Tubulin
Synapsin1
Neuronal PAS domain protein 4
Glycine receptor alpha1 subunit
Synaptotagmin 5
5-Hydroxytryptamine receptor 5A

F


Neuronal system
Potassium channels
Transmission across chemical synapses
Voltage gated potassium channels
Signaling by GPCR
GPCR downstream signaling
GPCR ligand binding
Cardiac conduction
Muscle contraction
Neurotransmitter receptors and postsynaptic signal transmission
Protein-protein interactions at synapses
Class A/1 (Rhodopsin-like receptors)
G alpha (q) signaling events
G alpha (s) signaling events
Neurexins and neuroligins
Interaction between L1 and Ankyrins
Transcriptional regulation by MECP2
Presynaptic depolarization and calcium channel opening
Nervous system development
Platelet calcium homeostasis
Peptide ligand-binding receptors
Regulation of insulin secretion
G alpha (i) signaling events
Immune system
Axon guidance
Neurotransmitter release cycle
ADORA2B mediated anti-inflammatory cytokines production
Serotonin neurotransmitter release cycle
Amine ligand-binding receptors
0
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20
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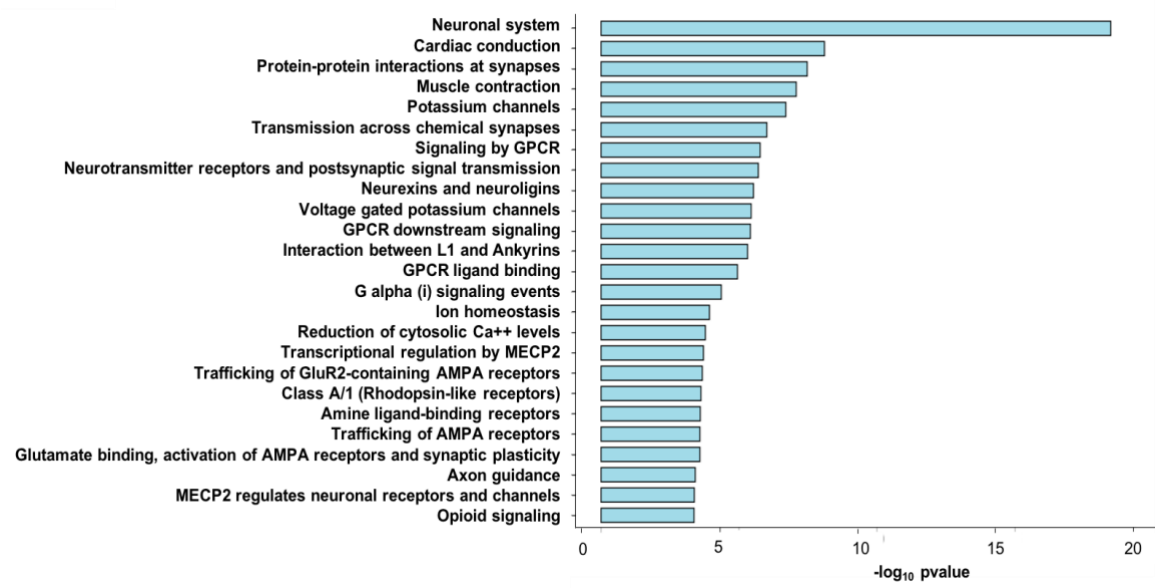
G


Neuronal system
Cardiac conduction
Protein-protein interactions at synapses
Muscle contraction
Potassium channels
Transmission across chemical synapses
Signaling by GPCR
Neurotransmitter receptors and postsynaptic signal transmission
Neurexins and neuroligins
Voltage gated potassium channels
GPCR downstream signaling
Interaction between L1 and Ankyrins
GPCR ligand binding
G alpha (i) signaling events
Ion homeostasis
Reduction of cytosolic Ca++ levels
Transcriptional regulation by MECP2
Trafficking of GluR2-containing AMPA receptors
Class A/1 (Rhodopsin-like receptors)
Amine ligand-binding receptors
Trafficking of AMPA receptors
Glutamate binding, activation of AMPA receptors and synaptic plasticity
Axon guidance
MECP2 regulates neuronal receptors and channels
Opioid signaling
0
5
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15
20
-log10 pvalue

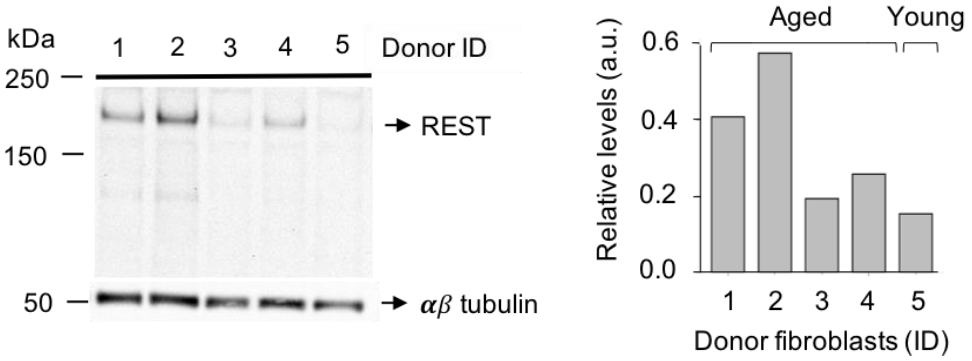
Figure 4. REST ChIP-Seq in human iNs and parental fibroblasts from 68y old donor (AG04349A, Coriell). Fib = Fibroblasts

A) Venn diagram of all REST ChIP-Seq peaks in aged Fib and iNs. B) Pie-chart showing fraction of RE1 and non-RE1 sites in Fib and iNs. C) Venn diagram showing overlap in RE1 containing peaks between the Fib and iNs. D) schematic of REST motifs (consensus full (top), left and right half sites (bottom)) used for motif analysis, adapted from McGann et al., 2021. E) Representative REST ChIP-Seq tracks showing peaks at the RE1 sites associated with the following neuronal genes - glycine receptor subunit alpha 1 (GLRA1), 5-Hydroxytryptamine Receptor 5A (HTR5A), Synaptotagmin 5 (Syt5), Neuronal pas domain protein 4 (NPAS4), Synapsin1 (Syn1) and βIII tubulin in Fib and iNs, identical scale range used for both cell types. F) & G) Reactome enrichment categories for genes associated with REST peaks containing RE1 motifs in F) Fib and G) iNs, *p* value calculated by Fisher's exact test and corrected by FDR (set to 0.05)

A

ID	Line	Age	Sex	Race	Biopsy Source
1	AG04349A	68	M	Caucasian	Arm
2	ND34769	68	F	Caucasian	Unknown
3	AG06959	67	M	Caucasian	Arm
4	AG16102	69	M	Caucasian	Arm
5	GM02171	22	F	Caucasian	Unknown

B



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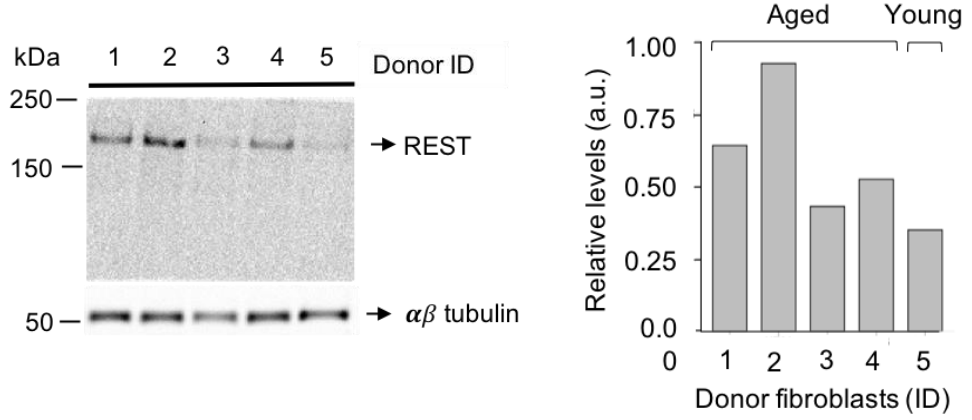

D

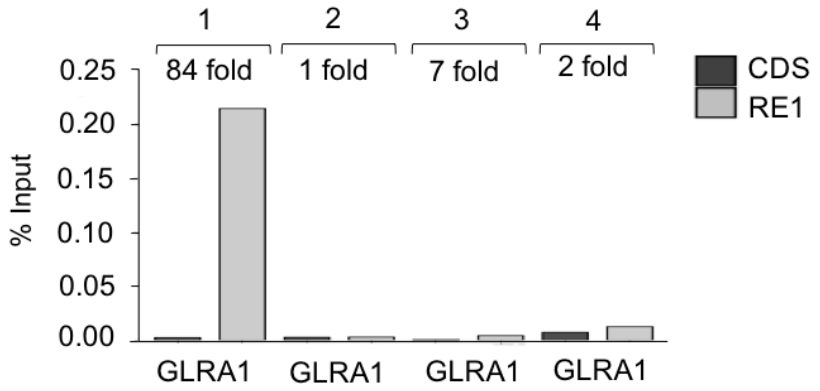

Figure 5. Inter-individual variability in REST expression and binding to chromatin

A) List of donor fibroblasts used in this experiment, all lines obtained from Coriell institute, B) & C) Western blot (Left) showing total REST protein in donor fibroblasts with B) C-terminal REST antibody and C) N-terminal antibody (Refer to materials and methods for antibody details), along with quantification (Right) of total REST levels normalized to loading control using anti-$\alpha\beta$ tubulin antibody, D) REST qChIP showing great inter-individual variability in amount of enriched REST at RE1 site associated with gene GLRA1. Primers for a region within the coding sequence of the GLRA1 (CDS) served as negative control for REST antibody pulldown.

Chapter 4: Study of the post-translational mechanisms regulating REST levels in human cells

Abstract

While the role of REST as a transcriptional repressor has been extensively studied, much less is known about the post-translational mechanisms regulating its cellular levels. In differentiating neural progenitors *in vitro*, REST levels decrease until it is completely removed from chromatin at terminal neuronal differentiation, which is required for the induction of a mature neuronal phenotype. This degradation of REST at the post-translational level, requires the activity of E3 ubiquitin ligase, β-TRCP, recruited by REST for targeting to the proteasome. Recently, a novel methylation modification of REST at lysine (K)494 by methyltransferase enzyme, EZH2, was proposed to regulate its levels in human cells, an intriguing model that had not been rigorously tested. Moreover, how methylation of REST at K494 worked together with the sites recruiting β-TRCP in the degradation of REST was unknown. Here, in my study, I re-examined the role of the K494 site in regulating REST levels in human cells by utilizing REST mutated at K494 (K494>A) and monitoring its degradation using *in vitro* protein degradation assays. I also investigated whether K494>A affected REST recruitment of β-TRCP for degradation. My findings show that the K494 site does not regulate REST levels in human cells, contradicting previous published work. Consequently, I also show that the K494 site does not regulate the recruitment of β-TRCP for its degradation. While my study eliminates a regulatory role for the K494 site, the approach and methods developed here may be used to investigate other potential regulatory sites in the human REST protein.

Introduction

In the previous chapter, I showed that reprogrammed iNs generated from human fibroblasts using miR-9/9* and miR-124 cannot be used to study REST function. Specifically, the highly variable REST profiles in the age-matched human donor fibroblasts, coupled with identical REST target genes between the donor fibroblasts and iNs, prevented me from utilizing this system any further to study REST. However, since I had already devoted a significant amount of time in developing this system, I decided to study a different aspect of the REST protein, which while still related to its regulation during the miRNAs-mediated reprogramming of fibroblasts to iNs, is independent of the aforementioned limitations in this system.

Protein degradation is a tightly regulated cellular process essential to maintenance of its homeostatic function. The half-lives of proteins can vary from minutes to days, with mammalian transcription factors typically having a short life span between 20 minutes to 2 hours (Curran et al., 1984; Lüscher & Eisenman, 1988). Polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), or PEST, are found in proteins typically targeted for rapid degradation (Rechsteiner & Rogers, 1996). Studies that have analyzed REST protein sequence have identified regions similar to PEST-like sequences, suggesting a need for rapid degradation of REST in certain contexts and possible post-translational regulation by proteolysis (Palm et al., 1998). While the role of REST as a transcriptional repressor has been widely studied, much less is known about the mechanisms regulating its protein stability.

Many proteins are subjected to changes at distinct amino acid side chains or peptide bonds shortly after their synthesis, commonly referred to as post-translational modifications (PTMs) (Ramazi & Zahiri, 2021). PTMs are highly varied in nature and can affect the activity, localization, stability and cellular interactions of proteins, thereby

increasing the functional diversity of the proteome. Three PTMs, ubiquitination, phosphorylation, and methylation, have previously been implicated in regulating the stability of REST (Refer to Chapter 1 section 1.4.2. for a more detailed background on the discovery of these PTMs in REST).

During *in vitro* neuronal differentiation of mammalian embryonic stem cells via intermediate neural progenitor cells, REST is ubiquitinated and undergoes degradation through a proteasomal pathway (Westbrook et al., 2008; Z. Huang et al., 2011). This process requires an interaction with β-TRCP, an E3 ubiquitin ligase belonging to the Skp, Cullin, F-box-containing complex (SCF) family. Degradation of REST by β-TRCP is essential for relieving its repression of neuronal genes, such as TUBB3, required for neuronal differentiation (Westbrook et al., 2008). Additionally, β-TRCP-mediated degradation of REST during neuronal differentiation requires REST to be phosphorylated by MAP kinases ERK1/2, demonstrating an interplay between two different PTMs in regulating REST protein levels (Nesti et al., 2014). Blocking this phosphorylation of REST in neural progenitors can result in highly stable REST protein levels due to impaired recruitment of β-TRCP, thereby disrupting their ability to differentiate into neurons. Hence, understanding the fundamental post-translational mechanisms that regulate REST protein stability is crucial for gaining insights into its role in mediating neuronal differentiation during development.

More recently, methylation was reported as a novel PTM in REST and shown to affect its stability in human cells (Lee et al., 2018). This study identified REST methylation mediated by EZH2, a methyl transferase enzyme, at amino acid residue K494. Mutation of K494>A, by presumably preventing methylation of REST by EZH2, made it unstable, thus facilitating faster degradation compared to wild-type (WT) REST protein. Further, this methylation-dependent control of REST levels was proposed to underlie the mechanism

by which neuronal fate acquisition was acquired during direct cellular reprogramming of human fibroblasts to iNs, using miR-9/9* and miR-124. This supposition is based on previous studies of neural progenitors showing that persistent expression of REST delays neuronal differentiation in vivo (Mandel et al., 2011). The findings related to REST methylation by EZH2 were intriguing to me for two major reasons. First, methylation of human REST protein was reported for the first time. Second, this study showed a direct interaction between REST and EZH2 in human cell lines by co-immunoprecipitation, which was not previously seen in mouse embryonic stem cells (McGann et al., 2014) suggesting a novel post-translational control of REST levels in terminally differentiated human cells.

Here, I decided to seek a better understanding of how the methylation of REST at K494 worked together with the β-TRCP-recruiting sites responsible for its degradation through the proteasome, which remained an open question. For this purpose, I first wanted to test in my own hands the role of the K494 site in regulating human REST protein levels. I used a human embryonic kidney cells (HEK cells or HEKs) based expression system, where I transfected WT- REST or REST mutated at the K494 site (K494>A) and performed *in vitro* protein degradation assays to monitor the kinetics of REST degradation. Then, I sought to investigate whether the K494 site in REST affected the degradation of REST through recruitment of β-TRCP.

Results

We choose HEKs to study the regulation of human REST protein for multiple reasons. First, HEKs are widely used as an expression system for studying recombinant proteins and contain the biochemical and molecular components required to generate a functional, mature protein (Thomas & Smart, 2005). Second, there is minimal endogenous REST expression in HEKs, making them suitable to study mutated forms of REST protein

by overexpression. Third, unlike primary human cell lines such as fibroblasts that have high variability in REST levels (Refer Chapter 3, Figure 5), HEKs provide a more controlled environment to study REST.

Mutation of the β-TRCP recruiting sites stabilizes human REST protein levels

Cycloheximide (CHX)-based chase assay is a widely used method to monitor protein degradation *in vitro* (Eldeeb et al., 2019). The assay involves the administration of the CHX compound to cells which globally inhibits protein synthesis by preventing translational elongation (Schneider-Poetsch et al., 2010). This is then followed by examining the levels of a target protein, such as REST, at different timepoints after normalizing its levels to a control stable endogenous protein, such as αβ-tubulin, in western blotting assays.

The goal of this experiment was to validate the use of CHX to monitor the kinetics of REST protein degradation. Here, I utilized a mutant version of human REST protein (β-TRCP-MUT REST) previously shown to stabilize its levels in HEKs (Guardavaccaro et al., 2008; Westbrook et al., 2008; Nesti et al., 2014). These mutations are in the C-terminal domain at the two sites known to recruit β-TRCP (Refer to Figure 1), and were generated in collaboration with Dr. Sayantani Ghosh-Dastidar in the Mandel lab. I transiently transfected HEKs with either WT- or β-TRCP-MUT- REST and treated them with CHX. Following treatment, I harvested whole cell protein lysates at fixed timepoints (1h, 3h and 5h) to determine REST protein levels over time. Figure 2A shows detection of REST following treatment of HEKs expressing either WT- or β-TRCP-MUT REST with CHX at different time-points. While WT-REST undergoes rapid degradation as detected by the significant drop in REST protein levels at 3h after treatment with CHX, β-TRCP-MUT REST levels continue to remain stable throughout the duration of the experiment (Figure

2B), similar to findings from previous publications (Guardavaccaro et al., 2008; Westbrook et al., 2008b)

Mutation of the previously proposed methylation site, K494 (i.e. K494>A), does not de-stabilize human REST protein levels

Next, I utilized the same CHX assay as above to test whether mutation of the K494 site in human REST protein (K494>A) de-stabilized it, as previously proposed by Lee et al (Lee et al., 2018). I transiently transfected HEKs with either WT- or K494>A- REST and treated them with CHX. Following treatment, I harvested whole cell protein lysates at fixed timepoints (1h, 3h and 5h) to determine REST protein levels over time. Figure 3A shows detection of REST following treatment of HEKs expressing either WT- or K494>A- REST with CHX at different time-points. Both WT-REST and K494>A- REST undergo rapid degradation as detected by the drop in REST protein levels at 3h after treatment with CHX with no significant difference in their degradation rates (Figure 3B). Of note, the reagents I utilized in this study i.e. WT and K494>A- REST expression vectors were the same constructs used in Lee et al's study, obtained from the lead author of this study, thus eliminating any experimental differences arising from using different molecular reagents. In conclusion, mutation of the K494 site (K494>A) did not affect the degradation of human REST protein in HEKs.

Mutation of human REST protein at K494 (K494>A) does not affect its interaction with EZH2

Since the difference in degradation rates of K494>A -REST compared to WT-REST in the study by Lee et al was suggestive of a loss of EZH2's methyltransferase activity, I wanted to test whether human WT-REST protein is in complexes with EZH2, and whether the mutation at K494>A eliminated or decreased its association with EZH2.

For this purpose, I first transiently transfected an expression vector encoding WT- or K494>A- REST fused to a hemagglutinin (HA) epitope tag in HEKs. After transfection, I immunoprecipitated both WT- or K494>A- REST using an anti-HA antibody, and then resolved the complex by SDS-PAGE, followed by a western blot probing for interaction of REST protein with EZH2 using an anti-REST and anti-EZH2 antibody (Figure 4A). I detected REST and EZH2 protein at their expected sizes of ~200kDa and ~98kDa, respectively, in lysates immunoprecipitated using anti-HA antibody, from both the WT- and K494>A- expressing HEKs, indicating mutation of REST at K494 (K494>A) does not prevent recruitment of EZH2 (Figure 4A). No REST or EZH2 was detected in lysates immunoprecipitated with an anti-IgG antibody, eliminating antibody-related non-specific interactions (Figure 4A). As another control, the levels of both WT- and K494>A- REST enrichment in the immunoprecipitation, relative to their corresponding input conditions, was significantly higher with the anti-HA antibody compared to the anti-IgG antibody (Figure 4B). Next, I quantified the amount of EZH2 in complexes with either WT- or K494>A- REST (refer methods section in chapter 2), and compared them to find out whether the mutation in REST (K494>A) affected EZH2 recruitment. Figure 4C shows no significant difference in the amount of EZH2 in complex with WT- or K494>A-REST, thus indicating mutation of K494>A site in human REST protein does not affect its interaction with EZH2.

The K494 site in human REST protein does not regulate the degradation of REST through its recruitment of β-TRCP

My previous results in Figure 3, showed no difference in the degradation rates of WT- and K494>A- REST. Based on this finding, my expectation was that the K494 site would not regulate the interaction of REST with β-TRCP for its degradation. In order to

test this hypothesis, I transiently transfected HEKs with human REST protein mutated at either just the β-TRCP-binding sites (β-TRCP-MUT-REST) or the β-TRCP-binding sites along with the K494 site (β-TRCP-MUT-K494>A- REST), and performed the CHX-chase assay. Figure 5 confirms my hypothesis, where I show stabilized REST levels when β-TRCP-binding sites are mutated with or without the K494>A mutation in REST protein. There was no significant difference in the stabilization of REST levels between the two conditions during the duration of the CHX-experiment (Figure 5B). Thus, these findings conclusively show no role for the K494>A site in regulating human REST protein levels.

Discussion

My findings agree with previously published work that REST is in complexes with methyltransferase enzyme, EZH2, in human cells. However, my findings could not replicate published work reporting that mutation of K494 in REST protein (K494>A) caused it to degrade faster than unmutated REST (WT) in human cells. This claim was based on the identification of a novel methylation modification at the K494 site, as a result of an interaction between REST and EZH2. Consequently, I also found that the K494 site does not affect REST degradation through its recruitment of the E3 ubiquitin ligase, β-TRCP.

Previous studies have shown degradation of REST is mediated through its interaction with β-TRCP, the substrate recognition subunit of the SCF family of E3 ubiquitin ligases (Guardavaccaro et al., 2008; Westbrook et al., 2008). A more detailed background of this discovery is provided in Chapter 1 section 1.4.2.1 under post-translational mechanisms regulating REST. Briefly, two independent groups identified one conserved sequence each, in the C-terminal domain of REST, to recruit β-TRCP. Mutation of these sequences (Refer Figure 1 schematic) in REST protein prevented recruitment of β-TRCP, subsequently leading to stabilization of REST levels in HEK cells, as detected in

cycloheximide (CHX)-based protein degradation assays. Indeed, here in my study, I was able to replicate these findings, where mutation of REST at the β-TRCP-recruiting sequences prevented the degradation of REST, resulting in significantly higher levels of REST protein compared to that of WT-REST during the duration of the CHX experiment (Figure 2).

EZH2 is a highly conserved protein, belonging to the SET-domain containing methyltransferase superfamily that catalyze lysine methylation in histones (Dillon et al., 2005). Studies on lysine methylation in both histone and non-histone proteins by EZH2, have demonstrated distinct effects on protein stability, leading to either protein stabilization or de-stabilization (Yang et al., 2009). For example, mono-methylation of RORα, an orphan nuclear receptor, by EZH2 leads to its recognition by DCAF1, a substrate recognition subunit of the DDB1-Cul4B ubiquitin ligase complex, in turn, leading to its degradation through the proteasome (Lee et al., 2012). Conversely, EZH2 methylation of Proliferating Cell Nuclear Antigen (PCNA), a scaffold for DNA polymerases during replication, stabilizes its trimerization, leading to its interaction with DNA polymerase (Peng et al., 2018). Similar to these findings, Lee et al proposed that methylation of REST by EZH2 at K494 stabilizes its levels, leading to potentially increased interaction of REST with its chromatin binding sites in human cells, particularly adult human fibroblasts. Their claim was based on observing faster decay of REST protein with K494>A mutation, compared to WT-REST protein in CHX-based protein degradation assays. However, a thorough examination of these results revealed this data was likely from a single replicate, and hence were not repeated or statistically evaluated. In contrast, I show that mutant REST (K494>A) does not significantly change its degradation kinetics compared to that of WT-REST by CHX-chase assays performed in three biological replicates (Figure 3).

EZH2 has been most extensively been studied in the context of its role as a catalytic subunit of the PRC2 complex, a conserved chromatin modifier with important roles in transcriptional silencing during development (Gan et al., 2018). Mass spectrometry approaches in mouse embryonic stem cells (mESCs) have previously reported REST interaction with members of the PRC2 complex, including EZH2. However, a direct interaction of EZH2 with REST was not detected by co-immunoprecipitation (Co-IP) assays in mESCs (McGann et al., 2014). Moreover, mESCs are pluripotent, as well a different species, which could manifest the differences in EZH2 recruitment by REST seen in terminally differentiated cells such as human fibroblasts, as shown in the study by Lee and colleagues (Lee et al., 2018). Here too, in my study, I was able to reproduce these results confirming REST interaction with EZH2 in HEKs overexpressing REST by co-immunoprecipitation assays (Figure 4). According to Lee et al's work, EZH2's interaction with human REST methylates it at the lysine residue (K494) in the conserved R-K-S amino acid sequence of the REST protein, which was chosen based on prior publications from other groups that showed the lysines within the R-K-S sequences of histone H3 and retinoic acid-related orphan nuclear receptor (RORα) to be the recipients of EZH2-mediated methylation (Cao et al., 2002; Lee et al., 2012). However, unlike the prior studies, such as the methylation of RORα by EZH2, Lee et al did not perform an unbiased mass spectrometric analysis of REST to confirm the methylation of K494 in REST protein. Instead, they utilized a pan-methyl-lysine antibody to detect methylation of REST in human cells, which bind methylated polypeptides, but are not specific to the amino-acid sequence targeted for methylation.

Lastly, upon comparing the degradation kinetics of REST mutated at both the β-TRCP-recruitment sites and K494>A, to that of REST mutated only at the β-TRCP-recruitment sites, I show no influence of the K494 site in stabilizing REST protein (Figure

5). In other words, the K494 site in REST does not affect REST degradation mediated by β-TRCP. This finding is consistent with my previous result (Figure 3), further validating that the K494 site in REST does not regulate its stability in human cells.

Collectively, my results conclusively contradict Lee et al's findings related to a role for the K494 site in regulating human REST protein levels. In doing so, my findings also raise important questions related to the proposed function of EZH2 as an effector of REST stability during the direct neuronal conversion of adult human fibroblasts into iNs using miR-9/9*-124. According to Lee et al's proposed model, transduction of miR-9/9*-124 in adult human fibroblasts downregulates USP7, a deubiquitinating enzyme specific for stabilizing EZH2, thus resulting in low EZH2 levels, which in-turn prevents it from methylating REST, leading to faster degradation of the REST protein and its subsequent removal from neuronal gene chromatin in the reprogrammed iNs. However, in my work, I have not been able to replicate both aspects of this model. First, REST is not completely removed from its binding to neuronal genes in the reprogrammed human iNs, as described in my previous chapter (Refer Chapter 3, results and discussion). Second, mutation of the proposed methylation site in human REST (K494) protein does not affect its stability in human cells (as described in this chapter). An important future step would be to re-evaluate the original model proposed by Lee et al, starting with re-examining whether REST is indeed methylated by EZH2 at the K494 site. This idea is discussed more in detail in the future directions section of the following chapter (Chapter 5).

Finally, while my study eliminates a role for the K494 site in regulating REST protein stability, there are other potential sites in human REST protein which can be explored in future studies in a similar manner. Figure 6 shows a schematic illustration of the human REST protein consisting of two new sites in REST that evolved in primates recently identified in the Mandel lab. The two sites, namely, the KEN- and D- box sites, short for K-E-N sequence of amino acids and destruction box, respectively, have

previously been reported to be capable of recruiting members of the Anaphase Promoting Complex/Cyclosome (APC/C) family of E3 ubiquitin ligases, which degrade proteins during cell cycle division (Fang et al., 1998; Pfleger & Kirschner, 2000). It is currently unknown how the KEN- and D-Box sites regulate the stability of human REST protein. Understanding their function in mediating REST protein degradation will provide invaluable insights into the regulation of human REST protein, with implications in human brain development. A more detailed discussion of the future directions related to human REST protein degradation through the APC/C is provided in the following chapter (Chapter 5).

Figures and figure legends

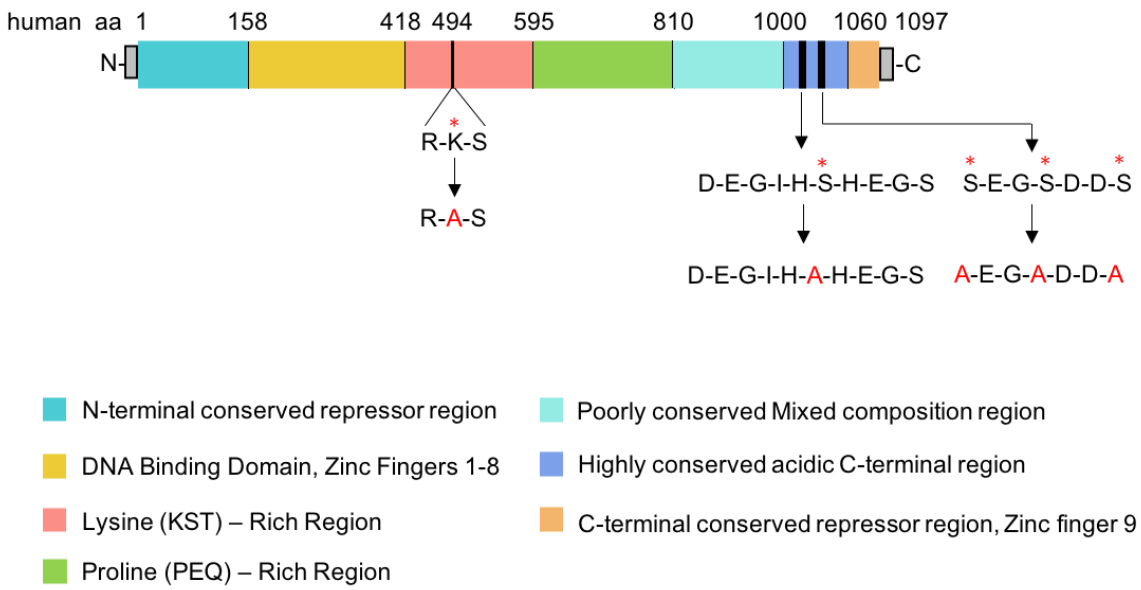

Figure 1. Schematic representation of human REST protein with the recently proposed methylation (K494) site and the two β-TRCP-recruiting sites (D-E-G-I-H-S-H-E-G-S and S-E-G-S-D-D-S). Mutations utilized in this study include K494>A and S1013,1024,1027,1030>A, marked with an asterisk (*).

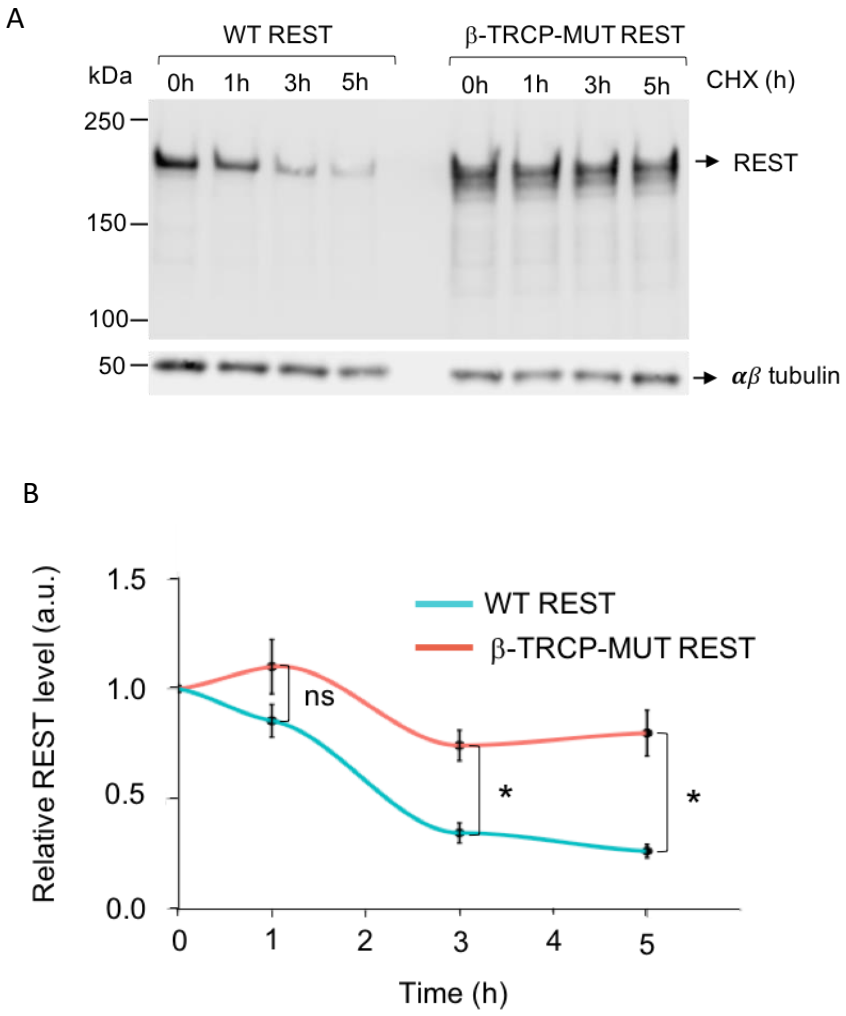

Figure 2. Mutation of the β-TRCP recruitment sites stabilizes human REST protein levels A) Representative western blot following CHX treatment (355uM or 100ug/ml) in HEKs transiently transfected with either WT-REST or REST mutated at the β-TRCP recruitment sites (β-TRCP-MUT-REST), B) Quantification showing relative REST levels normalized to loading control (αβ tubulin levels), error bars represent standard error mean (n= 3). *p<0.05, ns = not significant, Two sample student's t-test

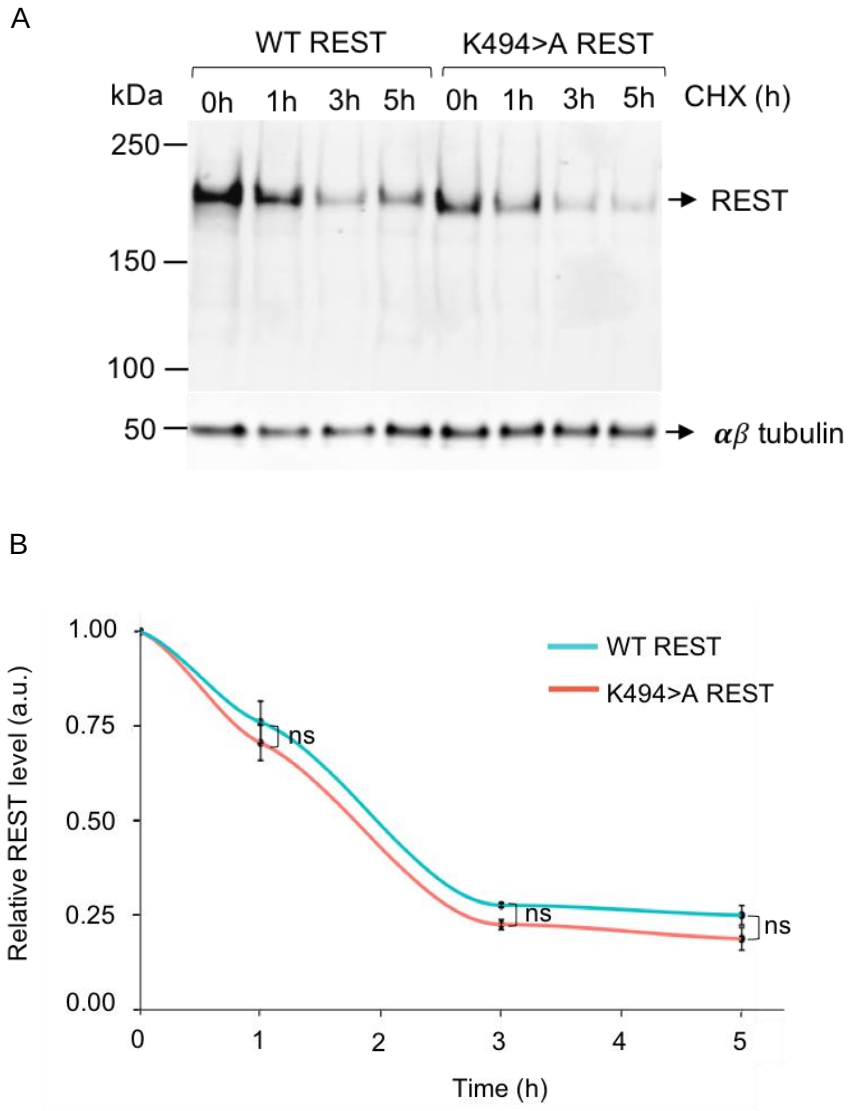

Figure 3. Mutation of the previously proposed methylation site, K494 (i.e. K494>A), does not de-stabilize human REST protein levels

A) Representative western blot following CHX treatment (355uM or 100ug/ml) in HEKs transiently transfected with either WT-REST or REST mutated at the K494 site (K494>A-REST), B) Quantification showing relative REST levels normalized to loading control (αβ tubulin levels), error bars represent standard error mean (N=3). ns = not significant, Two sample student's t-test

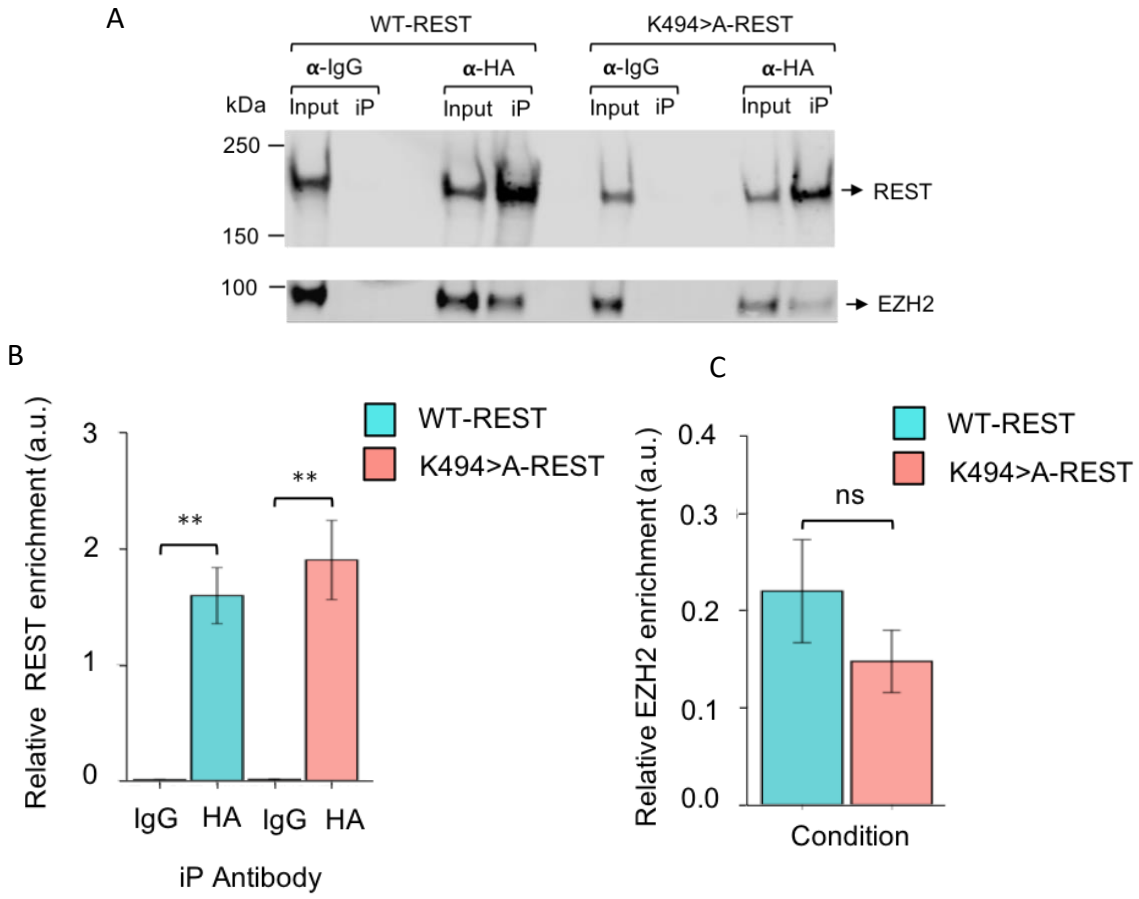

Figure 4. Mutation of proposed methylation site in REST protein (K494>A) does not affect its interaction with EZH2

A) Representative western blot following immunoprecipitation (iP) of overexpressed WT- or K494>A REST with anti -HA or anti -IgG antibody. Input lanes were loaded with 1% of lysates harvested prior to immunoprecipitation, B) Quantification showing enriched levels of iP'ed REST with HA antibody compared to IgG antibody (relative to corresponding input conditions) from HEK cells overexpressing either WT- or K494A- REST, C) Quantification showing relative enrichment of EZH2 in lysates iP'ed with anti-HA antibody. EZH2 levels were first normalized to amount of iP'ed REST to account for differences in iP efficiency. In B) and C), error bars represent standard error mean for N=3 biological replicates. **p<0.01, ns = not significant, Two sample student's t-test

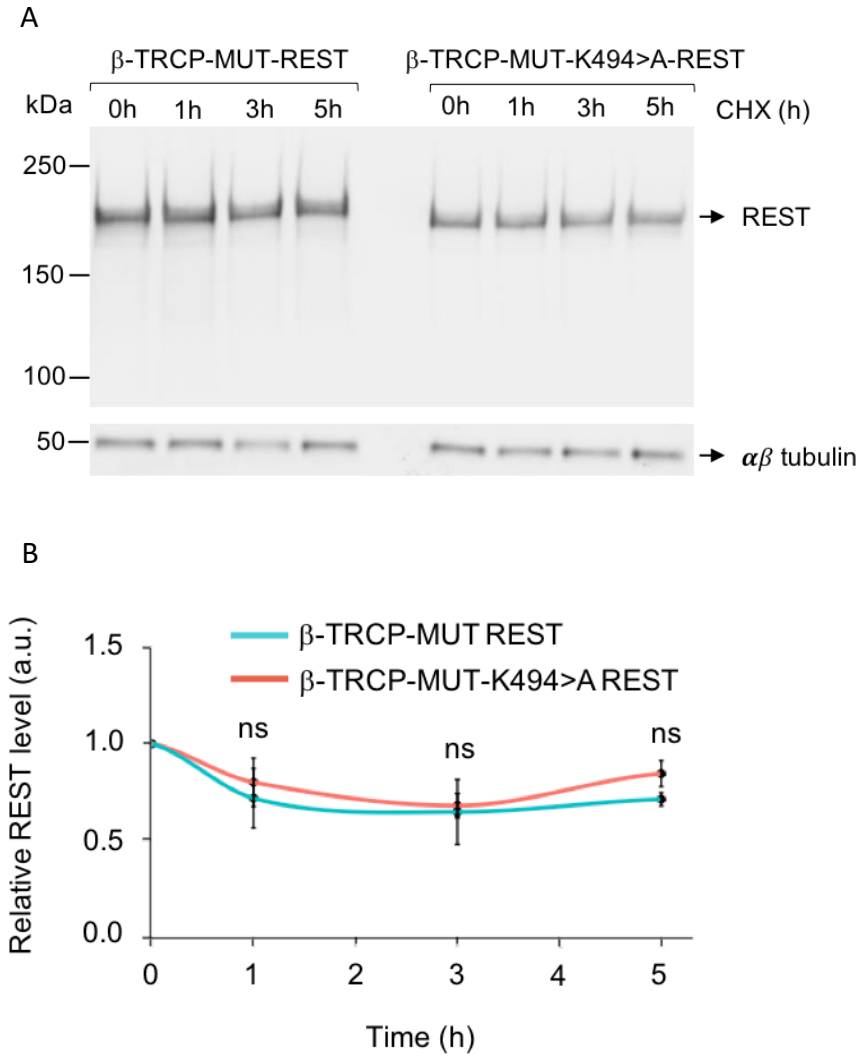

Figure 5. The K494 site in human REST protein does not regulate its degradation via recruitment of β-TRCP

A) Western blot following CHX treatment (355uM or 100ug/ml) in HEKs transiently transfected with either β-TRCP-MUT or β-TRCP-MUT-K494>A REST, B) Quantification showing relative REST levels normalized to loading control (αβ tubulin), error bars represent standard error mean (N=3) . ns = not significant, Two-sample student's t-test

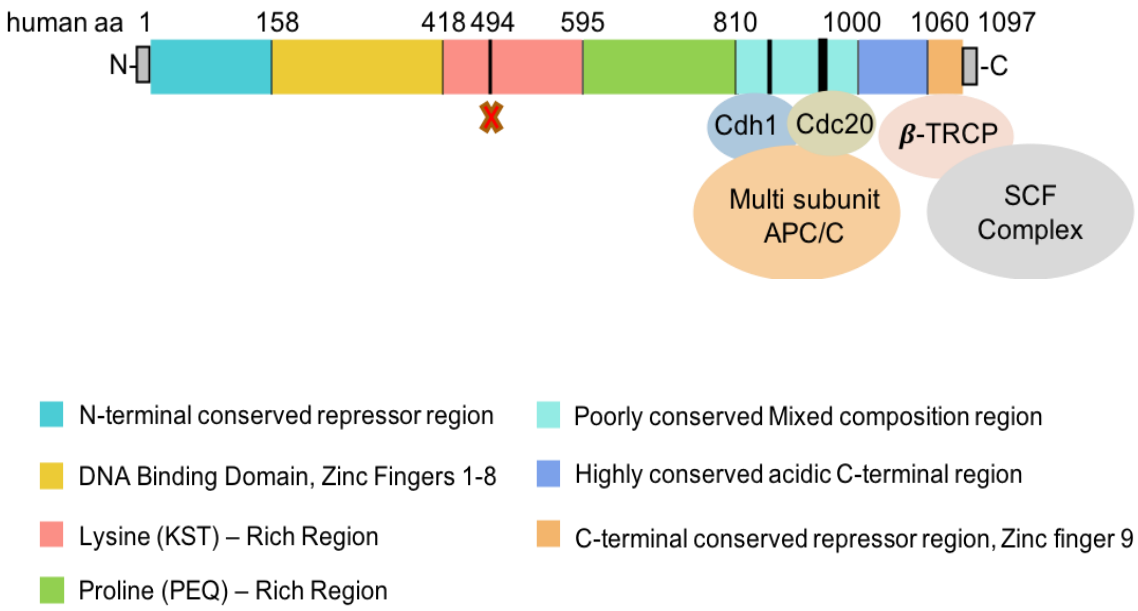

Figure 6. Cartoon of the human REST protein illustrating a proposed model of REST regulation in human cells. Based on the conclusions from this study, site K494 has been eliminated from regulating REST protein levels in human cells, marked with a red cross. Primate-specific sites KEN- and D-box sites, capable of recruiting APC/C complex through direct interactions with Cdc20 and Cdh1 are potential regulatory sites and may work in conjunction with the β-TRCP recruiting sites in regulation of REST in neural progenitors, to be explored in future studies.

Chapter 5: Final summary and future directions

In my dissertation research, I studied two different aspects of the human REST protein, one related to its role in neuronal aging, and the second, related to post-translational regulatory mechanisms controlling its levels in human cells. My findings have important implications for future research directions related to both *in vitro* aging models and for REST. In the following sections, I will briefly summarize my results and propose future studies that may provide a deeper understanding of the roles for REST in both the developing and aging human brain.

Limitations of *in vitro* neuronal models for studying aging, exemplified by studying REST

In Chapter 3, I profiled REST binding to chromatin using ChIP-Seq in human induced neurons (iNs), generated from dermal fibroblasts of an aged donor (68y) using microRNAs. While there are many studies of DNA methylation in aging with regard to the epigenetic clock, this is the first study to have characterized the genome-wide binding profiles of the epigenetic factor, REST, in *in vitro* aged primary human reprogrammed neurons. REST is an ideal factor for testing the utility of an *in vitro* neuronal system, because it regulates thousands of neuronal genes that together impart the terminally differentiated neuronal phenotype. I made two important findings: 1) that the differentiated human neurons, as defined by their REST binding site repertoire, are not likely terminally differentiated neurons as that of the in vivo neurons of the human brain. Not only was REST binding to canonical RE1 sites associated with neuronal genes, evident from the ChIP-seq analyses, but it was also likely repressing at least some of the target genes, evident from the recruitment of its chromatin remodeling co-repressor, CoREST, 2) it is difficult to conclude whether the fibroblasts from younger and older donors truly give rise to neurons with corresponding age-associated transcriptomes, as I found great variability

in the amounts of REST even among the fibroblasts from donors of the same age. Moreover, *in vitro* human neuronal aging models have been mostly studied in a global manner, such as analyses of the transcriptome, chromatin accessibility, DNA methylation status etc., as opposed to focusing on a single factor, such as REST. A careful retrospective examination of published work that prompted my studies indicates that there are inconsistencies between the different assays, particularly with regards to the use of cells from different donors for different assays and the number of biological replicates performed for an individual experiment. For example, in my own study, where I examined whether the mutation in K494 site in human REST protein regulated its stability, I performed the *in vitro* protein degradation assays in multiple biological replicates (n=3) and statistically evaluated the data before drawing any conclusions. However, previous published work (Lee et al., 2018) had only reported a single replicate experiment to derive conclusions as to whether the K494 site regulated REST protein levels in human cells.

Recently, two studies that utilized distinct ways to generate reprogrammed primary human neurons from somatic cells to study Alzheimer's disease (AD), a brain aging disorder, have speculated opposing roles for REST in AD (Meyer et al., 2019; Mertens et al., 2021). In the study by Meyer et al, the authors derived neural progenitors (NPs) from induced pluripotent stem cells (iPSCs) derived from dermal fibroblasts of sporadic AD (SAD) patients. SAD NPs exhibited reduced nuclear REST levels and consequently reduced binding at RE1 sites associated with neuronal genes, such as synaptic gene SNAP25, and sodium voltage gated channel gene, SCN3B, resulting in their higher expression, compared to that of healthy controls. Additionally, the SAD NPs were also shown to exhibit higher levels of early neuronal differentiation marker genes, such as DCX (doublecortin), compared to healthy controls. Collectively, based on these findings, the authors proposed that the SAD NPs exhibited an accelerated neuronal differentiation phenotype, due to a loss of REST function, ultimately resulting in the dysregulation of

neural gene networks underlying AD. In the other study by Mertens et al, they generated human iNs by direct reprogramming of dermal fibroblasts from AD patients (sporadic and familial) using a transcription factor-based approach (i.e. ectopic expression of neurogenin-2 and Achaete-scute homolog 1). Upon examining the transcriptomic landscape of the AD iNs, they identified downregulated expression of mature neuronal genes, such as the Glutamate ionotropic receptor, GRIK2, and an upregulation expression of genes related to an immature neural precursor state, such as Hes3. Motif analysis of the downregulated genes revealed an RE1 sequence amongst the majority of them, suggesting an increased REST repressor function underlying the altered transcriptional network of the neural genes in AD iNs. Collectively, based on the controversial findings described above, and from my own studies here in my dissertation, my conclusion is that iNs do not faithfully resemble mature neurons of the human aging brain, and is therefore not a good model to examine the role of REST during aging. Finally, while my research was in progress, other studies by members in the Mandel lab discovered that REST is likely preferentially expressed in glial cells of the human brain, such as astrocytes, oligodendrocytes, and microglia during aging, with low and dispersed expression in neurons. Because astrocytes are differentiated much later than neurons both in vivo and in *in vitro* reprogramming systems, I conclude from my work that human iNs-based culture systems might be best suited for looking at early events of neurogenesis.

Inter-individual variability of REST in primary human cells

In future studies on aging, I would consider using a different source of fibroblasts than the Coriell Institute. For example, primary human donor cells from the Baltimore Longitudinal Study of Aging (BLSA) collection can be considered. The BLSA is an initiative by the National Institute of Aging (NIA), wherein dermal cells from the same individual is

collected at several ages, along with a detailed record of information related to the donor's health. While investigators continue to make progress on understanding biological variation in primary human donor cells (Mirauta et al., 2020; Beekhuis-Hoekstra et al., 2021), utilizing cells from the same individual over their entire lifespan might be an alternative approach to minimize variability issues.

Methylation of human REST protein

In Chapter 4, I describe my findings from examining a recently reported potential post translational modification of REST (Lee et al., 2018), namely methylation at the lysine(K) 494 site, in regulating its levels in human cells. I found the study by Lee et al intriguing because their findings seemed to suggest that REST had evolved to be regulated through a novel post translational mechanism in human cells. I was able to replicate that REST is in complexes with EZH2 in human cells, as reported, and the basis for the methylation of REST. I was also able to replicate that the mutation of REST at its degron sites, which recruit an E3 ubiquitin ligase, β-TRCP, stabilized its levels, as previously published (Guardavaccaro et al., 2008; Westbrook et al., 2008; Nesti et al., 2014). However, by utilizing the same molecular reagents and biochemical techniques as that of Lee et al, I was unable to replicate that mutation of human REST protein at K494 (K494>A) changed the degradation profile of REST, compared to that of WT-REST, in a reproducible and statistically significant manner. Moreover, the addition of the K494>A mutation to REST already mutated at the β-TRCP recruitment sites did not significantly alter its stabilization, further validating my findings that K494 does not regulate human REST protein levels. An important follow-up study would be to determine directly whether human REST protein is methylated at the K494 site using an independent method, such

as mass spectrometry which will not only identify methylated residues but also localize the site of modification (Afjehi-Sadat & Garcia, 2013).

Primate-specific sites in REST protein as potential regulators of REST stability in human cells

While my study eliminated the K494 site in human REST protein as a candidate for post translational regulation, a similar *in vitro* and biochemical strategy can be applied to investigate other potential regulatory sites in human REST protein, as discussed below.

An analysis of the evolutionary history of REST indicated strong positive selection during primate and human evolution, particularly in the sites surrounding a region of variable number of tandem repeats (VNTR) (Mozzi et al., 2017). VNTR is a short DNA nucleotide sequence organized as a tandem repeat, whose length can vary amongst individuals and thus represent genetic variation. The authors identified 22 positively selected sites in non-human primate and human REST, all within a relatively large region surrounding the VNTR that also encode the PEST sequences i.e. peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T), that are proposed signal peptides targeted during protein degradation. The Mandel lab recently identified two newly evolved sites in primates REST protein, namely the K-E-N sequence of amino acids (KEN box) and the destruction box (D-box) (Refer Figure 6 schematic in Chapter 4), that had not been previously described. The KEN- and D-box are known to act as the substrate recognition motifs for recruitment of the Anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that functions during the cell cycle (Fang et al., 1998; Pfleger & Kirschner, 2000). Two APC/C activators, namely, Cdh1 and Cdc20, can directly bind to the KEN and D-box sequences in different cell types, and subsequently target the protein substrate for degradation through the ubiquitin protease system. As a cell

undergoes division, it goes through a sequence of cell-cycle phases, G0, G1, S, G2 and M. The transitions of the cell through these phases is governed by the sequential degradation of cell-cycle proteins, such as cyclins, carried out primarily by the Skp1/Cullin/F-box (SCF) and APC/C family of E3 ubiquitin ligases (Castroet al., 2005). It is already known that REST is degraded during the G2 phase of cell-cycle, by β-TRCP-SCF complex, which in-turn relieves its repression of the Mad2 gene that is required for transition to the M phase in HeLa cells (Guardavaccaro et al., 2008). Additionally, REST downregulation in neural progenitors close to the time they exit the cell cycle relieves REST repression of neuronal genes, thereby controlling the timing of terminal neuronal differentiation (Westbrook et al., 2008; Nesti et al., 2014). Moreover, in the in-vivo mouse brain persistent expression of REST during embryonic neurogenesis delays migration of radial glia, causing spatio-temporal defects in the transition of neural progenitors to neurons (Mandel et al., 2011). I initiated this work in collaboration with a Dr. Sayantani Ghosh-Dastidar, a postdoctoral scientist in our lab. Our hypothesis is that the APC/C mediated degradation pathway is important to downregulate REST levels during the generation of neural progenitors in non-human primates and/ humans, resulting in a large progenitor pool, leading ultimately to cortical expansion. Unfortunately, my preliminary experiments testing this hypothesis in transfected human embryonic kidney cells (HEKs), as a quick way to see whether deletion of the KEN- and D-box sites in human REST protein affected degradation rates of REST, did not show a predicted effect. Dr. Ghosh-Dastidar will be pursuing further experiments to test this hypothesis. In a methodological approach, she proposes to test for interaction between human REST protein and members of the APC/C, namely, Cdc20 and Cdh1, ideally through mass spectrometry analysis and co-immunoprecipitation assays. Then, the KEN- and D-box sequences in human REST protein can be mutated and their degradation profiles be monitored and compared to that of WT-REST protein using cycloheximide-based assays in human neural

progenitors. In mice, in-vivo introduction of the KEN- and D-box sites in endogenous REST could then be tested for cortical expansion. Ultimately, these studies will help gain a deeper understanding of the unprecedented roles for REST in the development of the human brain and perhaps shed light on mechanisms underlying the emergence of human cognition.

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