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The Role of UPF3B in Pluripotency and Differentiation

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The Role of UPF3B in Pluripotency and Differentiation

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Ada Le Shao

Committee in charge:

Professor Miles F. Wilkinson, Chair
Professor Jens Lykke-Andersen
Professor Alysson Muotri
Professor Amy Pasquinelli
Professor Tariq Rana

2015
The Dissertation of Ada Le Shao is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

University of California, San Diego

2015
DEDICATION

I thank my Lord and Savior for His miraculous saving work in my life. He has been faithful and gracious to me, most evidently in these past several years. During my years in San Diego, He has sustained me through the ups and downs of life and provided me amazing labmates, friends, and church family, who have been a huge blessing to me throughout graduate school.

I dedicate this dissertation to my parents, without whom I would not have been able to pursue my dreams and goals. Any achievements of mine were possible only because of their ceaseless dedication, support, care, and guidance. For the past six years, they have patiently endured holidays spent apart and limited visits home due to work and put up with my constantly vague answers to “when will you be done with school?” But not any longer…(almost) done!
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Chapter 3 and 4, in full, are being prepared for submission. The dissertation author is the primary investigator and co-first author on this publication.

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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

The Role of UPF3B in Pluripotency and Differentiation

by

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Doctor of Philosophy in Biomedical Sciences

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Professor Miles F. Wilkinson, Chair

The Nonsense-Mediated RNA Decay (NMD) pathway prevents the accumulation of potentially toxic proteins in the cell by targeting aberrant RNA transcripts with premature termination codons (PTCs) for decay. While originally for its RNA surveillance capacity, NMD was more recently discovered to target not only aberrant RNA transcripts but also normal, wild-type transcripts, regulating 5-20% of
the normal transcriptome. This, in turn, has led to the hypothesis that NMD, by fine-tuning gene expression, functions in orchestrating biological processes such as development.

Recent studies have identified mutations in the NMD factor gene, UPF3B, as the cause of syndromic and non-syndromic intellectual disability in human patients; these patients also frequently suffer from psychiatric and neurodevelopmental disorders, including schizophrenia, autism, and attention-deficit disorder. The role of NMD in neurodevelopment was further supported by work from our laboratory identifying the function of miR-128 mediated repression of NMD in neural differentiation.

For these reasons, I elected to study the role of the NMD factor UPF3B and microRNA regulation of NMD in neurodevelopment. In summary, I have identified a microRNA regulatory circuit in which the neurally enriched microRNAs, miR-132 and miR-9/-124/-128, repress the UPF2 and UPF3B branches of NMD, respectively. To examine the role of the UPF3B branch of NMD in neurodevelopment, I derived iPSCs and NPCs from patients with UPF3B-null mutations. Using these UPF3B-deficient iPSCs and NPCs, I obtained substantial evidence that UPF3B promotes pluripotency, while depletion or absence of UPF3B promotes differentiation.
CHAPTER 1: INTRODUCTION
INTRODUCTION

As a gene is expressed from its DNA blueprint, premature termination codons (PTCs) arise from mutations or can be introduced into the intermediate RNA transcripts through errors in transcription or splicing. If translated, these PTC-bearing transcripts would generate truncated, potentially deleterious or nonfunctional proteins. By targeting these aberrant RNA transcripts for decay, the Nonsense-Mediated RNA Decay (NMD) pathway prevents the accumulation of potentially toxic proteins in the cell.

While originally discovered in 1979 for and essential in its RNA surveillance capacity (Losson & Lacroute, 1979), NMD was more recently discovered to target not only aberrant RNA transcripts but also normal, wild-type transcripts, regulating 5-20% of the normal transcriptome (Mendell et al, 2004; Wittmann et al, 2006). This, in turn, has led to the hypothesis that NMD, by fine-tuning gene expression, functions in orchestrating biological processes such as development.

NMD Mechanism

NMD functions through several NMD factors, including the core factors UPF1, UPF2, and UPF3B. An additional set of factors that together function in NMD comprise the exon-junction complex (EJC,) which is deposited on exon-exon junctions after splicing and helps identify premature stop codons (PTC) that are located downstream of stop codons and thus trigger NMD (Le Hir et al, 2000a, 2000b, 2001). EJCs located upstream of the stop codon are displaced by the ribosome during
translation, whereas EJCs downstream of the stop codon escape this and thus flag the transcript for degradation. The EJC core consists of Y14, MAGOH, eiF4A3, and MLN51 and directly interacts with UPF3B, which through UPF2 forms a bridge to the central NMD factor, UPF1 (Gehring et al., 2003; Chamieh et al., 2007; Kadlec et al., 2004). Through a complicated choreography, which is still not fully understood, the NMD factor complex forms in a linear, sequential pathway. Following translation termination, UPF1 initiates formation of the SURF complex, which is comprised of UPF1, eRF1, eRF3 and SMG1 (Kashima et al., 2006; Kadlec et al., 2004). The UPF2 and UPF3B-mediated bridge facilitates the interaction of the EJC and the SURF complex, initiating SMG-1 phosphorylation of UPF1 and subsequently triggers NMD (Figure 1.1).

**NMD Branches**

While certain current models propose that NMD factors assemble in a seemingly inter-dependent and linear manner, there is evidence suggesting that NMD can still function in the absence of certain key factors. The requirement or, conversely, the dispensability of particular NMD factors defines distinct NMD branches apart from the canonical NMD pathway; these branches include: (1) the UPF2-independent branch, (2) the UPF2/RNPS1-dependent branch, (3) the EJC-independent branch, (4) the UPF3B-independent branch. (Gehring et al., 2005; Bühler et al., 2006; Chan et al., 2007) The UPF2 branch permits NMD activity by two routes: one in which Y14, MAGOH, and eiF4A3-initiated NMD occurs independent of UPF2 and a second in which NMD triggered by RNPS1 requires UPF2 (Gehring et al,
2005). Shortly after the discovery of the UPF2 branch, a second branch was reported, whereby degradation of immunoglobulin transcripts occurs in the absence of an intron downstream of the stop codon, suggesting the nonessential role of the EJC and thus an EJC-independent branch of NMD (Bühler et al., 2006). Lastly, our laboratory reported the UPF3 branch of NMD, in which certain substrates are degraded by NMD even in the absence of UPF3B while for other transcripts, UPF3B is essential to their degradation (Chan et al., 2007). Not surprisingly, these branches each regulate specific subsets of transcripts, rather than all NMD targets (Figure 1.2). While there have been attempts to reconcile the various branches and models of NMD (Ivanov et al., 2008; Metze et al., 2013), there is still no consensus on the exact choreography and mechanism of NMD. To add to that complication and perhaps even explaining the dispensability of certain NMD factors, our laboratory and others discovered a complex feedback mechanism that buffers NMD and allows NMD to continue function when certain factors are depleted (Huang et al., 2011; Yepiskoposyan et al., 2011a; Degtiar et al., 2015).

NMD Feedback and Regulation

Our work and that of another laboratory elucidated a cell type and tissue specific feedback mechanism that sustains NMD function when it is perturbed (Huang et al., 2011; Yepiskoposyan et al., 2011b). When the balance of NMD factor levels is disturbed, namely through depletion or loss-of-function of NMD factors, cells upregulate expression of unaffected NMD factors to compensate and maintain NMD activity. UPF1 and SMG7 are upregulated in response to UPF3B depletion in HeLa
cells, facilitating continued NMD function as evidenced by repression of known NMD substrates. Similarly, UPF3B-patient derived lymphoblastoid cells demonstrate regulation of not only UPF1 and SMG7 but also UPF2. Examination of various Upf3b-null tissues revealed a tissue-specific buffering system, wherein certain tissues show no change in NMD factor expression while others such as the brain and spleen upregulate Smg1 and Upf1/Smg6, respectively.

While NMD is in certain respects self-regulating by adjusting NMD factor expression in response to outside perturbation, as an indispensable regulatory pathway, it is also subject to external regulation. For example, MARVELD1, a tumor suppressor, was found to inhibit NMD by inhibiting the pioneer round of translation (Hu et al., 2013). Assessment of NMD in various tissues revealed that expression of NMD factors was tissue specific, suggesting that the strength of NMD varied on the specific cell/tissue context (Zetoune et al., 2008). Our laboratory identified a microRNA, miR-128, which regulates expression of the central NMD factor, UPF1 and MLN51 (Bruno et al., 2011). Highly conserved across vertebrate species, miR-128 represses UPF1 and MLN51 by 70% and is dramatically induced during brain development and neural differentiation. In neural stem cells, miR-128 induces neural differentiation, while UPF1 expression concurrently is reduced; this results in the upregulation of transcripts involved in neural processes, suggesting that miR-128 mediated repression of NMD promotes neural development (Figure 1.3).
Physiological Role of NMD

The role of NMD in neural development along with its regulation of specific normal transcripts leads to the hypothesis that NMD, by fine-tuning gene expression, functions in orchestrating biological processes such as development. In support of this hypothesis, deletion or mutation of genes encoding essential NMD factors cause embryonic lethality and developmental defects in a variety of vertebrate species.

In mice and drosophila, knockout of NMD factors UPF1, UPF2 and SMG1 (in mice only) is embryonic lethal (Avery et al, 2011; Metzstein & Krasnow, 2006; Rehwinkel et al, 2005; Medghalchi et al, 2001; Weischenfeldt et al, 2008; McIlwain et al, 2010). Knockdown of UPF1 and UPF2 in zebrafish causes severe developmental delays and patterning abnormalities (Wittkopf et al, 2009). In mice, UPF2 was found to function in hematopoetic stem and progenitor cell maintenance (Weischenfeldt et al, 2008) as well as liver development, function and regeneration (Thoren et al, 2010). Other NMD factors have also been extensively studied in various model systems.

Interestingly, a common theme amongst the physiological functions of NMD factors is neural development and function. In zebrafish, depletion of UPF1, UPF2, UPF3A, or UPF3B causes brain-patterning abnormalities, implying a role for NMD in development of the central nervous system (Wittkopp et al, 2009). In Drosophila, UPF2 functions in synaptic development and function (Long et al, 2010). In frogs and rats, EIF4A3 functions in sensory neuron development, touch response, neural crest-derived melanophores, overall development of neural-epidermal border development and synaptic strength and neuronal protein expression, respectively (Haremaki et al,
Magoh haplosufficiency in mouse causes microcephaly by impairing neural stem cell division suggesting that this EJC factor functions in embryonic brain development (Silver et al., 2010).

**NMD and Brain Development**

In line with NMD functioning in brain development, recent studies have identified mutations in the NMD factor gene, *UPF3B*, as the cause of syndromic and non-syndromic intellectual disability in human patients; these patients also frequently suffer from psychiatric and neurodevelopmental disorders, including schizophrenia, autism, and attention-deficit disorder (Tarpey et al., 2007; Laumonnier et al., 2010; Addington et al., 2011; Szyszka et al., 2012; Lynch et al.; Xu et al., 2012). To elucidate the specific function of NMD in brain development and neurological disorders, our laboratory generated a *Upf3b*-null mouse (Huang et al.). These mice have behavioral/sensory and molecular defects that in part recapitulate those observed in human patients. Like intellectual disability and schizophrenia patients, these mice have defects in fear-conditioned learning, pre-pulse inhibition, and olfaction; at the cellular level, these mice displays reduced dendritic spine density and immature spine morphology.
Figure 1.1 NMD factors assemble on the target mRNA transcript to elicit decay. The exon-junction complex is deposited on mRNA transcripts following splicing. NMD factors, including UPF1, UPF2, and UPF3B, are recruited to the target transcript. Upon assembly of the NMD factor complex, the mRNA is subject to degradation.
Figure 1.2 NMD consists of branches that each regulate a subset of NMD targets, and that all depend on the central factor, UPF1. The canonical NMD pathway utilizes all NMD factors, while specific branches function in the absence of particular factors, regulating only a subset of NMD substrates.
Figure 1.3 NMD is regulated by the microRNA, miR-128. miR-128 inhibits UPF1 during brain development, relieving repression of a battery of transcripts involved in neural development.
REFERENCES


Huang L, Lou CH, Chan W, Shum EY, Shao A, Stone E, Karam R, Song HW & Wilkinson MF (2011) RNA homeostasis governed by cell type-specific and

Huang L, Shum E & Wilkinson M Manuscript in Preparation.


CHAPTER 2: THE NEURAL MICRORNA-NMD CIRCUIT
INTRODUCTION

**microRNA Biogenesis and Mechanism**

Like NMD, microRNAs constitute a major post-transcriptional gene regulatory mechanism that fine-tunes gene expression. A massive array of newly discovered non-coding RNAs (ncRNA) with diverse functions continue to be discovered, as evidenced by the constant stream of papers identifying RNA molecules with novel characteristics and functions. microRNAs were amongst the first ncRNAs found to function in modulating expression of their target genes. A mere 22nt in size, microRNAs repress their target mRNAs by binding to their complementary regions in the 3’ UTR (Bushati & Cohen, 2007; Bartel, 2009).

microRNAs are encoded in intergenic regions or run antisense to or are located within the introns of host genes (Lagos-Quintana *et al.*, 2002). Once transcribed, the pri-miRNA transcript hosting the microRNA is processed to its shorter pre-miRNA form by DROSHA-DGCR8 and exported to the cytoplasm (Denli *et al.*, 2004; Gregory *et al.*, 2004). There, Dicer cleaves off the stem-loop, leaving the miRNA-miRNA* duplex (Chendrimada *et al.*, 2005). In most cases, the passenger strand, miRNA*, is degraded, while the mature miRNA is loaded onto the RNA-induced silencing complex (RISC), consisting of Dicer, Argonaute and other proteins necessary for its gene-silencing activity (Schwarz *et al.*, 2003; Du, 2005; Gregory *et al.*, 2005; Maniataki & Mourelatos, 2005).
Once loaded on the RISC, microRNAs repress their target mRNAs by 1) destabilizing them, 2) reducing their translational efficiency, or, in rare cases, 3) cleaving them (Behm-Ansmant & Izaurralde, 2006; Wu et al, 2006; Humphreys et al, 2005; Maroney et al, 2006; Petersen et al, 2006; Yekta et al, 2004).

**microRNA Expression and Function**

Conserved from plants to animals, microRNAs are critical gene expression regulators. Their spatially and temporally defined expression allow for the precise control of their target mRNAs, especially in processes requiring gene expression regulation within a narrow window of time, location, and magnitude, such as development. Widely expressed in all tissues, microRNAs have a diverse functional repertoire but are particularly critical in the development and function of the brain (Cao et al, 2006; Li & Jin, 2010; Fineberg et al, 2009; Gao, 2010; Schratt, 2009) where 70% of detectable microRNAs are expressed (Babak et al, 2004). Of those with tissue-specific expression, 50% are brain-specific or enriched.

Within the human brain, microRNAs exhibit highly differential, regionalized and temporal expression during development and function (Ziats & Rennert, 2014). Loss-of-function studies have demonstrated specific roles for microRNAs in development, including in neural development (Bushati & Cohen, 2007; Fineberg et al, 2009; Li & Jin, 2010). For example, mutations in Argonaute 2, which disrupts RISC repressive activity, block neural tube closure in mice (Liu et al, 2004). Conditional deletion of Dicer in various neuronal subtypes implicated the role of microRNAs in cortex development, dendrite maturation, and neurogenesis (Cuellar et
al, 2008; Davis et al, 2008; De Pietri Tonelli et al, 2008). Depletion of Dicer in mature neurons in adult mouse forebrain causes a short spike in learning and memory ability followed by rapid degeneration (Konopka et al, 2010). In this Chapter, I will examine how microRNA modulation of NMD guides neural development.
NMD, microRNAs, and Brain Development

The link between microRNAs and NMD was first established in 2010, when it was shown that Argonaute 2 inhibits NMD of mRNAs bound by cap-bind protein and exon-junction complexes (Choe et al, 2010). Later, our laboratory identified miR-128 as a repressor of NMD factors, *UPF1* and *MLN51* (Bruno et al, 2011). miR-128 mediated repression of NMD upregulates a battery of transcripts involved in neural differentiation and function, which is supported by evidence for the role of UPF3B in neural development (Huang et al). This study inspired me to examine how microRNA-mediated regulation of NMD affects developmental and physiological processes. The multiple layers of post-transcriptional gene regulation permit a highly specific and nuanced fine-tuning of downstream target expression. Given the strong evidence for the role of both microRNAs and NMD in brain development and neurological disorders, I hypothesized that these two pathways closely interact to promote proper neurodevelopment.

As neurodevelopment necessitates regulation of well-defined gene subsets in a spatiotemporally controlled manner, I considered how this might be accomplished by regulation of NMD and its downstream substrates. As our previous work examined how miR-128 represses NMD as a whole by downregulating the central factor, *UPF1*, I sought to examine how regulation of other NMD components, either by regulating branch-specific, multiple or highly targeted NMD factors might permit a more refined,
responsive, adjustable regulation of NMD and its targets. This sort of regulation would accommodate the refined gene expression necessary during neurodevelopment and neural processes.
RESULTS

Identification of microRNAs that regulate NMD factors in the brain

To identify microRNAs regulating NMD, I performed \textit{in silico} screening for microRNAs predicted to the target mouse “core” NMD factors, focusing on well-conserved microRNAs and seed matches, as these are more probably \textit{bona fide} microRNA-target mRNA interactions (Friedman \textit{et al}, 2009). While much improved in identifying positive hits, sequence-based target prediction algorithms exhibit a high false positive rate, as many factors in addition to sequence complementarity impact microRNA regulation of a given target. I took advantage of the availability of multiple target prediction programs and chose three by which to perform the \textit{in silico} screening, each using different criteria to generate predictions. TargetScan requires exact complementarity between the seed sequence and seed matches and also factors in conservation and complementarity beyond the seed match (Lewis \textit{et al}, 2003, 2005). The TargetScan algorithm predicted that \textasciitilde 375 microRNAs target NMD factor mRNAs. Comparison with miRanda predictions, which calculates free energy of the heteroduplex but allows some matching in the seed match region (Betel \textit{et al}, 2008), generated 87 microRNAs predictions consistent across the two the algorithms. I further filtered the potential microRNAs using \url{www.microcosm.org}, which utilizes the miRanda algorithm but considers additional factors to impose greater stringency. From these microRNAs, I eliminated microRNAs that were not predicted to target at
least two NMD factor mRNAs, as microRNAs targeting multiple NMD factors would be more likely to repress NMD activity.

**Characterization of microRNAs and NMD factors selected for further study**

Finally, I prioritized the microRNAs based on relevance in human NMD targets and selected the final microRNAs for study based on their known roles in neural development and disease (Table 2.1). Based on the criteria above, I focused my efforts on the microRNAs, miR-9, -124,-128, 132, and 485-5p, all of which have well-characterized functions in neural development and processes and are also implicated in a number of neurological disease states (Table 2.2). Based on studies in rodent and primate tissues, these microRNAs have expression consistent with that of their function in brain development and neural differentiation (Babak et al, 2004; Bak et al, 2008; Barad, 2004; Faghihi et al, 2010; Kim et al, 2004; Lagos-Quintana et al, 2002; Miska et al, 2004; Olsen et al, 2009; Sempere et al, 2004; Thomson et al, 2004; KRICHEVSKY et al, 2003; Smirnova et al, 2005).

miR-9 is a brain-specific microRNA that in expressed at low level during early development and increase during embryonic development, declining postnatally. Its expression is moderate to high in most brain regions tested. miR-9 inhibits proliferation and promotes neural progenitor migration and differentiation in the cortex, directing cells towards the neural lineage (Delaloy et al, 2010; Leucht et al, 2008; Krichevsky et al, 2006; Conaco et al, 2006; Zhao et al, 2009; Shibata et al, 2008; Packer et al, 2008).
miR-124 too is a brain-specific microRNA that is initially expressed at low levels but increases during embryonic development and plateaus at birth. It is the most abundant and ubiquitously expressed microRNA in the brain, comprising 25-48% of all microRNAs there (Lagos-Quintana et al, 2002) where its expression is highly restricted to neurons. miR-124 inhibits neural progenitor proliferation, promotes gene transcription directed towards neuronal lineage commitment and directs neurite outgrowth (Conaco et al, 2006; Visvanathan et al, 2007; Cheng et al, 2009; Makeyev et al, 2007; Yoo et al, 2009; Yu et al, 2008).

miR-128, like miR-9 and miR-124, is also a brain-specific microRNA, whose expression starts low but increases late in development until adult. Its expression is high in the cortex and hippocampus and is restricted to neurons. In glioblastoma cells, miR-128 inhibits proliferation, suggesting its role in inhibiting neural progenitor proliferation (Godlewski et al, 2008). It is upregulated in autism spectrum disorders (Abu-Elneel et al, 2008).

miR-132 is predominantly expressed in embryonic stem cells and is also enriched in the brain. Its expression peaks during early development and decrease until adult, with enrichment in the developing forebrain. miR-132 functions in neurite outgrowth and remodeling of mature dendrites (Vo et al, 2005; Wayman et al, 2008). By regulating FMRP and MeCP2, miR-132 is implicated in neurodevelopmental disorders, Fragile X syndrome and Rett syndrome (Edbauer et al, 2010; Klein et al, 2007).
miR-485-5p is a brain-enriched microRNA, with highest expression in the cortex and hippocampus. It regulates neurite outgrowth, dendritic spine density and synapse formation (Cohen et al, 2014, 2011).

Intriguingly, the expression pattern of these microRNAs during brain development is reciprocal to that of certain core NMD factors, such as UPF1, UPF2, and UPF3B (Figure 2.1). While UPF1 and UPF2 steadily decreased during mouse embryonic development, UPF3B displayed a sharp increase between E10.5 and E14.5, a period of brain development marked by intense neurogenic activity in the cortex. MLN51 expression, in contrast, was relatively stable during embryonic development.
Table 2.1 Selected microRNAs and their predicted NMD factor targets. Based on *in silico* screening, neurally expressed microRNAs targeting at least two NMD factors were selected for validation.

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<td>RNPS1</td>
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Table 2.2 microRNAs and NMD factors involved in neural development and disease. From in silico screening results, microRNAs were selected for further study based on 1) involvement in neural development and function 2) target NMD factors being implicated in neural function and neurodevelopmental disorders.

<table>
<thead>
<tr>
<th>microRNAs</th>
<th>Neural Function/Disease</th>
<th>NMD factors</th>
<th>Potential Regulatory microRNAs of implicated NMD factors</th>
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<tr>
<td>miR-124</td>
<td>Synaptic plasticity</td>
<td>UPF2</td>
<td>miR-128, 132, 485-5p</td>
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<tr>
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<td></td>
<td>EIF4A3</td>
<td></td>
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<tr>
<td>miR-485-5p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-9</td>
<td>Neuronal differentiation</td>
<td></td>
<td></td>
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<tr>
<td>miR-124</td>
<td></td>
<td></td>
<td></td>
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<td>miR-128</td>
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<td>miR-124</td>
<td>Neurite outgrowth and maturation</td>
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<td>miR-485-5p</td>
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<tr>
<td>miR-128</td>
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<td>UPF3B</td>
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<td>miR-132</td>
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<td></td>
<td>Rett Syndrome</td>
<td></td>
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<td>miR-132</td>
<td>Schizophrenia Intellectual Disability</td>
<td>UPF3B</td>
<td>miR-9, -124, -128, 485-5p</td>
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Figure 2.1 NMD factors display varying expression profiles during embryonic mouse brain development. Expression of NMD factors, *Upf1*, *Upf2*, and *Upf3b* decreases over the course of mouse embryonic brain development, while *Mln51* expression remains unchanged. *Upf3B* expression spikes between E12.5 and 14.5, a period marked by intense neurogenesis in the cortex. NMD factor expression normalized to RPL19.
Validation of putative NMD-regulating microRNAs

To validate the putative NMD-regulating microRNAs, I first identified appropriate cell lines for loss- and gain-of-function studies. I assessed a range of human and mouse cell lines at varying confluencies for expression levels of microRNAs selected for study. From these, I then selected cell lines with low basal expression of a particular microRNA for gain-of-function experiments and lines with medium-to-high expression for loss-of-function experiments (Figure 2.2).

To assess microRNA repression of the putative targets, the 3’ UTR of the putative target were sub-cloned downstream of the luciferase reporter gene. I co-transfected these reporter constructs into cells along with either the microRNA mimic or inhibitors to test both gain- and loss-of-function. To demonstrate that the microRNAs act directly on their targets, I performed site-specific mutation studies with microRNAs confirmed via Luciferase assays to have an effect on their respective target NMD factors. If the repression is a direct effect of the microRNA on its target, mutations in the seed match sequence of the putative targets should abolish repression. As the final confirmation, I assessed the effect of these microRNAs on endogenous protein levels by transfecting cells with microRNA mimic or inhibitor and performing Western blotting. Lastly, to ensure that microRNA regulation of their respective target NMD factors, I assessed expression of NMD substrates.

As expected, the majority of the putative microRNA-target NMD factor did not hold up after extensive validation attempts. While the target prediction algorithms factor in sequence complementarity, binding energies, and conservation amongst other
considerations, microRNA activity also depends greatly on context and expression of microRNAs and their respective targets. After assessing nearly all the possible microRNA and putative NMD factor targets in multiple conditions and cell lines, I found in most cases either a complete lack of target repression or drastically variable results, both of which imply no interactions leading to repression. While many of the microRNAs display mild repression of their targets, this sort of regulation may not be sufficient to elicit strong downstream effect. However, I did reproducibly find that four microRNAs did effect NMD factor expression, based on luciferase reporter analysis. I found that UPF2 and RNPS1 were consistently downregulated by miR-132, supporting the idea of branch-specific regulation of NMD. UPF3B, a predicted target of several microRNAs, was repressed by miR-9, 124, and 128, which is interesting as one factor is the target of multiple microRNAs, suggesting that precise modulation of its expression may be critical to proper brain development.
Figure 2.2 microRNA expression varies between mammalian cell lines. microRNA expression in different mouse and human cell lines was assayed by Taqman qPCR analysis. Expression levels were normalized to U6. P19 cells were assayed in non-differentiation conditions and with retinoic acid (RA) to induce neural differentiation.
miR-132 regulation of the UPF2 branch

To assess regulation of the UPF2-RNPS1 branch of NMD, I assessed repression of UPF2 and RNPS1 luciferase reporters by miR-132. Both UPF2 and RNPS1 were downregulated by the miR-132 mimic and upregulated by the miR-132 inhibitor in mouse and human cell lines (Figure 2.3, 2.4). Mutation of the miR-132 binding site in UPF2 resulted in loss of regulation, suggesting that the effect is direct (Figure 2.3, 2.4). Endogenous expression of UPF2 and RNPS1 decreased with the miR-132 mimic, further validating the repressive effect of miR-132 on the UPF2-RNPS1 branch of NMD (Figure 2.5).

While confirming the miR-132 repression of UPF2 was a start, it was essential then to assess downstream effects of this repression, most importantly to establish an impact on NMD activity. Assessing a battery of classical and UPF2 branch substrates, I found in the mouse cell line LMTK, that UPF2 substrates previously identified as UPF2-dependent in were largely unaffected (Gehring et al, 2005; Weischenfeldt et al, 2008). In HeLa cells, while a few of the classical NMD substrates were upregulated by miR-132, UPF2-dependent substrates were downregulated or unaffected. Substrates identified in UPF3-knockdown HeLa cells showed modest upregulation, indicating that NMD is likely affected (Figure 2.6). Together, these results suggested that miR-132 represses UPF2, which in turn affects downstream NMD targets that depend UPF2.
Figure 2.9 miR-132 represses UPF2 reporter expression. A and B. Cells transfected with miR-132 mimic and inhibitor had reduced and increased levels, respectively, of a Firefly luciferase reporter construct of the UPF2 3’ UTR normalized to a co-transfected Renilla luciferase control. C. Mutation of the miR-132 binding site resulted in loss of repression.
Figure 2.4 miR-132 represses RNPS1 reporter expression. A. LMTK cells transfected with miR-132 mimic had reduced expression of a RNPS1 3’ UTR luciferase reporter. B. Cells transfected with miR-132 inhibitor conversely demonstrated increased expression of the RNPS1 reporter. Firefly luciferase reporter expression was normalized to a co-transfected Renilla luciferase construct.
Figure 2.5 miR-132 represses endogenous UPF2 and RNPS1 expression. LMTK cells were transfected with miR-132 mimic. *UPF2 and RNPS1 mRNA levels were reduced, as assayed by qPCR normalized to GAPDH.
Figure 2.6 miR-132 mediates upregulation of NMD substrates. A. NMD substrate expression in HeLa was assayed by qPCR analysis after transfection with miR-132 mimic. UPF2-dependent substrates (increasing UPF2-dependence indicated by arrow) were generally unaffected or downregulated. B. Substrates identified in UPF2-knockdown are unaffected by miR-132 mimic transfected in P19 cells. qPCR results normalized to GAPDH.
*miR-9, -124, -128 regulation of UPF3B*

Based on reporter analysis, UPF3B was regulated by several neural-specific microRNAs. The UPF3B luciferase reporter construct was downregulated by the miR-9, -124, and -128 mimic (Figure 2.7A, 2.8A, 2.9A). Conversely, it was upregulated by the microRNA inhibitors in both mouse and human cell lines (Figure 2.7B, 2.8B, 2.9B). Mutation of the miR-9 binding site in the UPF3B 3’UTR seemed to ablate miR-9 repressive ability on UPF3B (Figure 2.7C). Similarly, mutation of the miR-128 binding site in UPF3B while not completely eliminating function modestly reduces its repressive function (Figure 2.9C). At the endogenous level, miR-9 strongly reduces *UPF3B* at the mRNA level while miR-124 and miR-128 modestly reduce its expression (Figure 2.10).

To assess the downstream effect of microRNA activity on UPF3B, I evaluated expression of UPF3B-dependent and independent substrates in cells transfected with miR-9, -124, and -128 inhibitors (Figure 2.11). Surprisingly, the NMD substrates tested were largely unaffected by miR-128 inhibitor, even though it also targets central NMD factor, UPF1. One explanation is that UPF1 is already expressed far in excess and any increase in its expression through miR-128 inhibition has no further impact on its NMD activity. In the cases of miR-9 and miR-124, there was reduced expression of select classical NMD substrates, particular *ATF3* and *ASNS*. Nicely fitting with their identification as UPF3B-independent substrates, a subset of NMD targets were unaffected or in some cases even increased in expression with miR-9, -124, and -128 inhibition.
These data together confirm that these three microRNAs together repress UPF3B expression, raising the hypothesis that they work synergistically to regulate UPF3B and ultimately impact NMD activity. Interestingly, the putative binding sites of these three microRNAs on the UPF3B 3’UTR are nearly adjacent to each other, further supporting the idea that these microRNAs cooperate in repressing UPF3B.
Figure 2.7 miR-9 represses UPF3B reporter expression. A. miR-9 transfected cells downregulated UPF3B 3’ UTR firefly luciferase reporter. B and C. Mutation of the miR-9 binding site ablates repression by miR-9 in P19 cells. Firefly luciferase reporter normalized to co-transfected renilla luciferase construct.
Figure 2.8 miR-124 represses UPF3B reporter expression. A. miR-124 mimic transfected cells downregulate UPF3B luciferase reporter. B. miR-124 inhibitor transfected HeLa cells upregulate UPF3B luciferase reporter. Firefly luciferase reporter normalized to co-transfected renilla luciferase construct.
Figure 2.9 miR-128 represses UPF3B reporter expression. A. miR-128 transfected cells have reduced UPF3B 3’ UTR firefly luciferase reporter expression. B. Mutation of the miR-128 binding site ablates repression by miR-128 in P19 cells. C. miR-128 transfected LMTK cells repressed UPF3B reporter construct. Firefly luciferase reporter normalized to co-transfected renilla luciferase construct.
Figure 2.10 miR-9, -124, and -128 repress endogenous UPF3B expression. Endogenous UPF3B is downregulated in HEK293 cells transfected with miR-9 mimic and in LMTK cells transfected with miR-124 and -128 mimics. Expression levels normalized to GAPDH.
Figure 2.11 miR-9, -124, and -128 upregulated a subset of NMD substrates
A. HeLa cells transfected with miR-9, -124, and -128 mimics displayed little or no change in classical NMD substrate expression; B. the effect was similar for presumably UPFB-dependent substrates identified in UPF3-knockdown HeLa cells.
Synergy between microRNAs

Interestingly, the miR-9, -124, and -128 binding sites reside adjacent to one another on the UPF3B 3’ UTR, raising the possibility that they cooperate to suppress UPF3B expression (Figure 2.12). To assess whether miR-9, -124, and -128 cooperatively regulate and thus more strongly repress UPF3B, I assessed the impact of these microRNAs, individually and in combination, on endogenous UPF3B and NMD substrate expression (Figure 2.13). In the case of ATF3, miR-9 inhibitor most dramatically reduced ATF3 expression relative to miR-124 and miR-128. The combination of miR-9 with either miR-124 or miR-128 but not the three together slightly increased repression of ATF3 relative to miR-9 alone. In contrast, ASNS was most reduced in expression with the combined activities of miR-9, -124, and -128, or miR-124 alone, but not with double combinations. Perhaps best demonstrating the synergistic effect of the three microRNAs, GADD45B and TBL2 expression changed only with combination of the three microRNA inhibitors but not with any of them individually. UPF3B-independent substrates, NAT9 and PANK2, were unaffected by any of the microRNA inhibitors, either individually or in combination. However, other NMD substrates, including “UPF3B-independent” ones, did change in expression level with certain combinations of these microRNA inhibitors. While these “UPF3B-independent” substrates were originally identified in knockdown studies, our laboratory has found that NMD activity and NMD factor dependence is cell type and context specific. Based on these data and what we know about context specific regulation of NMD, it would appear that in certain situations, these
microRNAs did cooperate on some level to repress UPF3B as assessed by expression of its targets.
miR-9 | miR-124 | miR-128
---|---|---
Hsa | AGGCAGCCAAAGACGACACG-UUAAGCAUCCAGAGUGCCUUCAGGGCA----AAG-AUAGAGA-----GAAAGGAGCCUUGCUGGGG |  |
Ptr | AGGCAGCCAAAGACGAC--G-UUGGGAUCCAGAG--------------GCA----AAGAAUGAGA-----UAAAGGGGCCUUGCUGGGG |  |
Mml | AGGCAGCCAAAGACGACACG--UUAAGCAUCCAGAGUGCCUUCAGGGCA----AAGAAUGAGA-----GAAAGGAGUCUUUCUGGGG |  |
Rno | AGGCAGCCAGUGCCUAGUGCCUAGAGUGCCUUCAGGGCA----GAGGAACGAGAAGCAAGAAGGUGGGG |  |
Gga | AGGCAGCCAAAGACGCGUUGA--CUACGCAAUCCAGAGUGCCUUCAGGGCAAGGAAAGGAACAAAGG----AGGAAGGGGGCCAUUGCCUGGGG |  |
Xtr | UAUAUACCAAAGAAACAAA--UUCAGCAAUCC---AGUGCCUUCAGAGG---AAGGAUCUGAGCAGGCGAAAGAGG--- |  |

**Figure 2.12** miR-9, -124, and -128 binding sites are adjacent on the UPF3B 3' UTR. The close proximity of the miR-9, -124, and -128 binding sites suggest that together these microRNAs can more strongly repress UPF3B.
Figure 2.13 Some microRNA combinations synergistically regulate NMD substrates. HeLa cells were transfected with miR-9, -124, and -128 inhibitors, individually and in combination. (A-E) Classical NMD substrates responded to certain NMD inhibitor combinations (F-J). Similarly for “UPF3B-independent” substrates. Normalized to GAPDH.
Feedback between microRNAs and targets

microRNAs are subject to regulation through feedback mechanisms, which act to either negatively regulate or positively reinforce microRNA expression (Chang et al., 2004; Johnston et al., 2005; Li & Carthew, 2005). Interestingly, miR-9, -124, and -132 were all reported to operate in feedback loop with their targets and regulatory factors. miR-9 and -124 both operate in feedback loops that function in a switch between the neural progenitor and neural differentiation fates (Visvanathan et al., 2007; Sun et al., 2007). MeCP2 in similar fashion represses miR-132, which would otherwise inhibit MeCP2, which functions in learning and memory and when mutated causes Rett Syndrome (Im et al., 2010). Furthermore, depletion of UPF1 results in upregulation of microRNA targets, demonstrating that interplay between the NMD and microRNAs pathways (Jin et al., 2009). Based on these data, I considered the possibility that this sort of feedback mechanism might also be at play between these NMD factors and their respective repressor microRNAs. Another laboratory member, Dr. Chih-Hong Lou, in fact independently corroborated my hypothesis in mouse neural stem cells, where he found knockdown of UPF1 induces miR-9, -124, and -128 expression (Lou et al., 2014). He found similarly that Upf3b-null mNSCs also demonstrate upregulation of miR-9, -124, and -128 relative to control. This feedback mechanism in a way functions as a negative or inhibitory “positive-feedback loop,” in which both the target NMD factor and the regulatory microRNA mutually repress each other.
Figure 2.14 microRNAs and NMD factors operate in a feedback circuit. UPF1 and UPF3B are repressed by neurally expressed microRNAs that are in turn repressed by UPF1 and UPF3B. This double feedback mechanism can serve to lock in a NMD factor expression state.
MATERIALS AND METHODS

Cell Culture and Transfection

All cell lines were cultured in DMEM and 10% FBS (except for P19, which was cultured in MEM-alpha and 10% FBS) and grown at 37°C and 5% CO2. Cells were seeded at 40,000 cells/well on 24-well plates the day before transfection. Reporter constructs and/or microRNA mimics/inhibitors (purchased from Ambion) were incubated with Lipofectamine 2000 in Opti-Mem and added to cells according to the Lipofectamine 2000 protocol. microRNA mimics/inhibitors were used at a final concentration of 20pmol/ml. Cells were harvested 24 hours post-transfection.

TaqMan Assay for microRNAs

Cells were harvested with Trizol Reagent for RNA isolation according to the Trizol Reagent protocol. Reverse transcription and TaqMan qPCR were performed according to TaqMan microRNA assay (TheromoFisher) protocol. microRNA expression was normalized to U6 expression.

Luciferase Reporter Assays

3’ UTRs of target NMD factors were cloned into the pMIR-REPORT vector. Site-directed mutations were introduced in 2-base pair segments across the binding site. Cells were seeded in 24-well plates at 40,000/well the day before transfection. microRNA mimics/inhibitors, firefly luciferase reporter construct, and a Renilla luciferase control construct were transfected at concentrations of 20pmol/ml, 100ng/ml, and 10ng/ml, respectively. Cells were harvested 24 hours post-transfection with passive lysis buffer after a PBS wash. Activity of the firefly and Renilla
luciferase was quantified through addition of Luciferase Assay and Stop&Glo Reagent available from Promega; light emission was measured by luminometer after addition of each luciferase substrate. Firefly luciferase measurements were normalized to Renilla luciferase readings.

**Gene Expression Analysis**

Cells were transfected with 20pmol/mol of microRNA mimic/inhibitor and harvested with Trizol Reagent (Thermo Fischer) for RNA isolation 24 hours post-transfection. RNA isolation was performed according to the Trizol Reagent protocol. RNA was reverse-transcribed with iScript Transcriptase (Bio-Rad), and qPCR analysis was conducted with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) according to protocol.
ACKNOWLEDGEMENTS

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REFERENCES


Huang L, Shum E & Wilkinson M Manuscript in Preparation.


CHAPTER 3: GENERATION OF UPF3B PATIENT-DERIVED IPSCS
INTRODUCTION

Numerous NMD factor-null animal models demonstrate the importance of NMD in early development and specifically in brain development. Deletion or mutation of genes encoding essential NMD factors cause embryonic lethality in mice models and developmental defects in a variety of vertebrate species; these defects primarily impact the brain but also the heart (Wittkopp et al, 2009; Haremaki et al, 2010). Studies in various animal model systems, including flies, zebrafish, and mice have shown that NMD functions in brain development and neuronal maturation (Tarpey et al, 2007; Laumonnier et al, 2010; Addington et al, 2011; Szyszka et al, 2012; Lynch et al; Xu et al, 2012). Deletion of core NMD factors, upf1, upf2, or upf3b in zebrafish produces defects in brain patterning, while depletion of Magoh and Eif4a3 in rodent animal models impairs neuronal maturation and neural stem cell proliferation, causing microcephaly (Silver et al, 2010; Giorgi et al, 2007).

Patients with NMD Deficiencies

In support of the neurological phenotypes observed in NMD factor depleted animal models, several studies have recently demonstrated an association between NMD factor mutations/copy number variations with various neurological and neurodevelopmental disorders. Mutations in EJC component, RBM8A, have been associated with TAR syndrome (Alachkar et al, 2013), which also frequently presents with craniofacial abnormalities and intellectual disability. A noncoding expansion in EJC component, EIF4A3, has been identified in patients with Richieri-Costa-Pereira
Syndrome, a craniofacial development disorder that is associated with learning disabilities (Favaro et al, 2014). Copy-number variations of UPF2, UPF3A, SMG6, RNPS1 have been described in patients with diverse forms of neurodevelopmental disorders (Nguyen et al, 2013).

One NMD factor that has drawn particular interest is UPF3B. In the past 8 years, eleven families have been identified with mutations in UPF3B (Table 3.1). These mutations in UPF3B have been identified as the cause of syndromic and non-syndromic intellectual disability in human patients; these patients also frequently suffer from psychiatric and neurodevelopmental disorders, including schizophrenia, autism, and attention-deficit disorder. Apart from the shared underlying neurodevelopmental deficits in these patients, the clinical presentation of these patients makes studying the role of UPF3B in these disorders of particular interest.
Table 3.1 UPF3B patients identified to date. Since the first report of UPF3B mutations in intellectual disability patient, 7 more families have been identified worldwide. Patients present with varying degrees of intellectual disability with or without syndromic features and frequently present with other neurodevelopmental/psychiatric disorders such. (Tarpey et al, 2007; Laumonnier et al, 2010; Addington et al, 2011; Szyszka et al, 2012; Lynch et al; Xu et al, 2012)

<table>
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<tr>
<th>Year</th>
<th>Country</th>
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<th>ID Severity</th>
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Identification of first UPF3B Patients

Since the original 2007 report of the first intellectual disability patients with mutations in UPF3B, seven more affected families have since been identified. Despite the recent interest in this gene and its role in cognitive impairment, intellectual disability caused by mutations in UPF3B is rare, as only 11 affected families have been identified worldwide. These patients are affected by impaired cognition as defined by an IQ below 70 and often present with syndromic features, such as abnormal craniofacial development and digit malformation. Interestingly, the disease phenotype of these patients is highly variable in terms of severity of intellectual disability, presence of syndromic features, and comorbidity with other neurological and psychiatric disorders. A more detailed description of these affected patients is provided in Table 3.1.

Evidence for Role of UPF3B in Neurodevelopment

Behavioral tests on Upf3b-null mice generated by our laboratory have revealed that these mice have impaired learning and memory, defective pre-pulse inhibition, and abnormal olfaction (Huang et al), all of which are also observed in intellectual disability and schizophrenia patients (Moberg et al, 1999; Hessl et al, 2009). Furthermore, our analysis has revealed significant reduction in dendritic spine density as well as immature and improper synaptic morphology in specific regions of Upf3b-null mouse brains. Neural stem cells isolated from these Upf3b-null mice exhibit impaired or delayed neural differentiation and also expressed increased levels of the early neural markers, Sox2 and Nestin. An analogous phenotype is observed in the
olfactory epithelium, where stem cells of the olfactory epithelium accumulate and are delayed in their development into mature olfactory neurons (Eleen Shum, Wilkinson laboratory, unpublished observations).

In a seemingly contradictory finding, the Gecz laboratory reported that depletion of UPF3B in mouse neural stem cells promotes self-renewal and delays their differentiation (Jolly et al., 2013). They found that UPF3B promotes neuronal maturation, as its depletion in hippocampal neurons results in reduced axonal length and increased arborisation of dendrites. Finally, they found that loss of UPF3B also results in upregulation of clinically relevant target transcripts in a neural cell specific manner.

Modeling of neurodevelopmental disorders using induced pluripotent stem cells

Since the revolutionary report of reprogramming human adult somatic cells back to a pluripotent stem cell state (Takahashi et al., 2007), the field has made great strides in capitalizing on this technology to study neurodevelopmental disorders. As the technology has improved, with robust protocols that boast of >2% efficiency and no integration of the potentially oncogenic reprogramming genes, there is now the possibility not only of studying these disorders but harnessing these cells as potential cell-based therapies.

With widespread adoption throughout many disease research areas, induced pluripotent stem cells (iPSCs) have been particularly impactful in the area of neurodevelopmental and psychiatric disorders (Onder & Daley, 2012; Mattis & Svendsen, 2011; Brennand & Gage, 2012; Kim et al., 2012; Dolmetsch & Geschwind,
2011; Marchetto et al, 2011; Chailangkarn et al, 2012). They have been successfully used to model and explore potential drug treatments for Rett syndrome, Fragile X, and schizophrenia, amongst many others (Ananiev et al, 2011; Kim et al, 2011; Cheung et al, 2011; Marchetto et al, 2010; Urbach et al, 2010; Sheridan et al, 2011; Brennand et al, 2011; Yoon et al, 2014; Chiang et al, 2011). While this approach has its limitations, being an in vitro system that cannot fully recapitulate the complexity of neural development and function, it is the only way to model and study these disorders using patient-derived cells. As such, it is a powerful tool to examine the underpinnings of these disorders on a molecular and cellular level. In this chapter, I report the generation and preliminary characterization of iPSCs derived from UPF3B patients.
PROJECT RATIONALE AND DESIGN

To examine the role of UPF3B in maintaining pluripotency and regulating neural development, ultimately with the goal of determining how the gene mutation causes the observed cognitive defects in human patients, I elected to harness the capabilities of patient-derived induced pluripotent stem cell technology, which promises several advantages to traditional, animal-model approaches.

Patient-specific iPSCs:

- are human cells
- are easily manipulated, as an in vitro culture system
- can recapitulate development unobservable in post-mortem samples or animals
- have been successfully used to model neurodevelopmental disorders
- elucidate patient-specific characteristics
- can be used as a model system to test potential drug candidates or treatments
RESULTS

Description of Patient Fibroblasts used in this Study

Patient and control dermal fibroblasts were obtained through our clinician collaborator, Dr. Jozef Gecz, who provided me with fibroblasts from three affected families.

Family 1

Two affected brothers were identified in Ireland (Lynch et al) with a two-base pair deletion in Exon 7 (c.697_698delAG,p.Arg223fs*30) that causes a frameshift and truncated protein that is targeted by NMD. The older brother, from whom we obtained dermal fibroblasts, was clinically diagnosed with intellectual disability, pervasive developmental disorder, attention-deficit disorders, and autism. He displayed an Asperger’s-like personality, a moderate case of mental retardation, as well as craniofacial dysmorphisms and kidney dysplasia. His younger brother, from whom we hoped to obtain dermal fibroblasts but ultimately were unable to, carried the same UPF3B mutation and was diagnosed with intellectual disability with speech delay and autistic features. He displayed macrocephaly and kidney dysplasia. The carrier mother, whose dermal fibroblasts are the familial control for my study, is of normal intellectual capacity and shows no sign of craniofacial dysmorphisms. She displays 100% X-chromosome inactivation skewing for the normal UPF3B allele. Despite having the same UPF3B mutation, these brothers display a clinically very
disparate phenotype. The majority of analyses were conducted with fibroblasts and iPSCs derived from the carrier mother and older brother.

*Family 2*

Two affected brothers were assessed in Australia (Tarpey *et al*, 2007). With two unaffected brothers and one unaffected sister, these two brothers were found to carry a deletion in Exon 9 of UPF3B (867_868delAG, G290fs*2). This deletion causes a frameshift and truncated protein targeted by NMD. Both brothers are clinically diagnosed with Lujan-Fryns syndrome (or marfanoid habitus syndrome), which is a form of syndromic X-linked mental retardation that presents with mild to moderate intellectual disability, pronounced facial dysmorphisms, and frequently psychiatric disorders. One brother displays severe intellectual disability while the other has a milder form.

*Family 3*

In the first report of UPF3B association with psychiatric disorders, two brothers from the US were ascertained to have a four base pair deletion in Exon 7 (C.83_686delAAGA, p.Q228fsX18) that results in a frameshift and truncated protein subject to NMD (Addington *et al*, 2011). In this family, the older brother was diagnosed with childhood-onset schizophrenia, autism, pervasive developmental disorder, and attention deficit and hyperactivity disorder. The younger brother, harboring the same mutation, was evaluated for autism, pervasive developmental disorder, and attention deficit and hyperactivity disorder and also displayed congenital pulmonary stenosis. At the time of publication, he had not experienced any psychotic
symptoms indicative of schizophrenia. The unaffected carrier mother displays a 74:26 X-chromosome inactivation skewing for the normal UPF3B allele in her peripheral blood cells.

Characterization of Patient and Control Fibroblasts

Prior to reprogramming, the patient derived and UPF3B-null dermal fibroblasts were characterized for UPF3B/NMD factor and NMD substrate expression (Figure 3.1). Interestingly, fibroblasts from the three families displayed unique NMD factor expression profiles. The specific mutations generate a premature stop codon, which subjects the \textit{UPF3B} mRNA to NMD, dramatically reducing \textit{UPF3B} expression in all cases. The expression levels of other NMD factors, however, varied tremendously across the families. This is not surprising as \textit{UPF3B} knockdown was shown previously to cause upregulation of other NMD factors depending on the cell/tissue type (Huang \textit{et al}, 2011; Yepiskoposyan \textit{et al}, 2011). This feedback mechanism among NMD factors is thought to buffer NMD activity such that even with perturbation, NMD can continue to function. This may explain why in the patients, including Family 1 shown in Figure 3.1, NMD substrates for the most part were not affected. In fact, in certain cases, NMD substrates were expressed at lower levels in patient fibroblasts than control. Between families, there was no clear-cut NMD substrate expression pattern, potentially indicative of both the varying levels of NMD factors and difference in genetic background/basal gene expression.
CRISPR-derived UPF3B knockout fibroblasts

To ascertain the role of UPF3B without the confounding affects of genetic background, I chose to reprogram UPF3B-knockout fibroblasts generated using the CRISPR approach. The Keith Joung laboratory at Massachusetts General Hospital helped with the design of three gRNA constructs targeting Exon 1 of UPF3B to induce NMD and ablation of UPF3B expression. The Alysson Muotri laboratory at UCSD provided me with normal male human dermal fibroblasts, which I nucleofected with Cas9 and the three gRNA constructs. I then reprogrammed the fibroblasts nucleofected with each gRNA construct.

Characterization of UPF3B knockout fibroblasts

The constructs used to generate the UPF3B CRISPR-based knockouts did not have a selection marker, and so I performed the analysis on a presumably mixed population of fibroblasts to identify which gRNA constructs caused a cut in UPF3B and consequently an indel in that region. Despite numerous assay optimization efforts, I could not validate that the CRISPR constructs/Cas9 indeed cut at the gRNA target site. While in a few rare instances, it appeared that a mismatch indicative of Cas9 cleavage or an indel was present in the target area, this result was not reproducible (Figure 3.2). Because I could not definitively select a fibroblast “line” containing the desired indel at the UPF3B locus, I decided to proceed with reprogramming using all three nucleofected fibroblast “lines” and later select for clones with UPF3B knockout.
Figure 3.10 Fibroblasts from Family 1 (Ireland) UPF3B patient have low UPF3B expression and altered levels of some NMD substrates. A. *UPF3B* expression assessed by qPCR (normalized to GAPDH) is reduced in patient fibroblasts relative to control fibroblasts. Patient fibroblasts harbor a nonsense mutation that subjects *UPF3B* mRNA to NMD. B. Classical NMD substrates are largely unaffected, while UPF3B-independent substrates are in fact downregulated.
Figure 3.211 CRISPR knockout was assessed by T7EI assay. A. post T7EI treatment, Lanes 1-2: PCR controls, Lanes 3 & 7: empty vector control; Lanes 4-6 and 8-10: potential knockout fibroblasts generated from 3 unique targeting constructs; faint bands visible in empty vector controls and knockout clones. B. previous results not reproducible. Lanes 1-8: annealed PCR product; Lane 1&5: empty vector control; Lanes 2-4 and 6-8: potential knockout clones, Lanes 10-17 same clones with T7EI enzyme treatment.
Generation of iPSCs

Family 1 iPSCs were generated through the Sanford Burnham Stem Cell Core using an episomal introduction of pCXLE-Oct4/shP53, pCXLE-Sox2/Klf4 and pCXLE-LMyc/Lin28. 6 control and 12 patient clones were generated.

Together with the Louise Laurent laboratory, I also reprogrammed all fibroblasts from the three families and fibroblasts nucleofected with the three UPF3B-targeting constructs. We opted for retroviral transduction of pMXs hOct4, hSox2, hKlf4, hMyc to increase efficiency of reprogramming. The results of the reprogramming are summarized in the table (Figure 3.3B). Unfortunately, due to an autoclave malfunction, the clones derived in the second reprogramming were lost due to contamination. Some experiments were conducted in clones salvaged from contamination through extensive antibiotic and antifungal treatment.
Figure 3.3 Patient and CRISPR-KO fibroblasts were reprogrammed into 200+ iPSC clones. A. Fibroblasts from 3 patient families and fibroblasts nucleofected with 3 unique CRISPR-knockout constructs were reprogrammed into iPSCS. Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) were introduced retrovirally. B. Colonies emerged for all lines between Day 18 and 30.
Validation of iPSCs

Karyotype

Control and patient iPSCs from Family 1 were karyotyped by Cell Line Genetics and have apparently normal karyotypes (Figure 3.4). One control and one patient iPSC clone could not be karyotyped and so were assessed for chromosomal aberrations through aCGH array.

DNA-Fingerprinting

DNA-fingerprinting was performed by Cell Line Genetics to validate the iPSC were derived from the parental fibroblast lines. Analysis revealed that all the control and patient iPSC were derived from their respective parental fibroblasts. Their short tandem repeat (STR) profile not match any cell lines from the Cell Line Genetics Library, which gives confidence that contamination from other cell lines was not present (Figure 3.5).

Teratoma

The three control and three patient lines were injected into SCID mice and monitored for teratoma formation. Only liquid filled tumors were isolated from the mice. However, all six lines could generate embryoid bodies (described in Chapter 4) that expressed lineage markers for ectoderm, endoderm, and mesoderm.

CRISPR Validation

Due to the large number of clones to screen, I opted to assess iPSC for evidence of CRISPR activity by gel shift mobility assay, which operates on the premise that indels located within the area of interest generate a loop structure and
therefore run slower on an acrylamide gel. However, despite many attempts, no clones displayed any evidence for indels located in the targeted region of UPF3B.
Figure 3.4 iPSCs generated from Family 1 have normal karyotypes. G-banded karyotype analysis was performed on all control and patient clones. All clones have apparently normal karyotypes. Representative images of control (A) and patient (B) iPSC karyotypes are shown.
Figure 3.5 iPSCs from Family 1 originate from parental fibroblasts. Short-Tandem Repeat (STR) 100 analysis of parental control and patient fibroblasts/iPSCs was conducted to verify cell identity. Analysis of 15 loci demonstrated that fibroblasts and iPSCs share the same STR profile, indicating that iPSCs originate from parental fibroblasts.
Characterization of iPSCs

**UPF3B and NMD Factor Expression in iPSCs**

Similarly to the fibroblasts, the patient iPSCs expressed UPF3B at ~20% the level of control iPSCs. However, unlike fibroblasts, *UPF1* and *UPF2* expression were both upregulated in patient iPSCs. However, this increased expression of *UPF1* and UPF2 alone was apparently insufficient to maintain expression of NMD substrates at control levels. Three classical NMD substrates, *ATF4*, *GADD45B*, and *GAS5* are upregulated in the patient lines relative to control, indicating impairment of NMD (Figure 3.6A).

Comparing NMD in fibroblasts relative to iPSCs, *UPF1* expression is relatively constant between the fibroblasts and iPSCs in both control and patient lines, while *UPF2* increases (Figure 3.6C-D). NMD substrates *ATF4* and *GAS5* both increase dramatically in iPSCs, particularly in the already NMD-impaired patient iPSCs. *GADD45B* expression, for unknown reasons, drops to near undetectable levels. Given the upregulation of the other NMD substrates, particularly in UPFB-deficient iPSCs, one could speculate that NMD is weaker in iPSCs relative to the more differentiated fibroblasts (Figure 3.6B-D).

**UPF3B Rescue**

To ascertain that the changes in NMD substrate expression were in fact due to UPF3B-deficiency, I exogenously expressed UPF3B from a lentiviral vector in the patient iPSCs. *UPF3B* was roughly 2.5-fold higher than control iPSC levels, and NMD substrate expression returns to levels typical of control cells. This, together
with data showing downregulation of classical (but not UPF3B-independent) NMD substrates in patient iPSCs, demonstrated that patient iPSCs could be rescued with exogenous UPF3B expression (Figure 3.7).

*Expression of Pluripotency Factors*

To assess proper induction of pluripotency, I examined expression of pluripotency markers in the reprogrammed iPSCs at both the RNA and protein level. Apart from *c-MYC*, all markers were dramatically induced in iPSCs relative to fibroblasts. Both control and patient iPSCs stained positive for pluripotency markers, OCT4 and NANOG, indicating successful reprogramming (Figure 3.8).
Figure 3.6 NMD in patient iPSCs is impaired. A. qPCR analysis reveal that patient iPSCs express significantly lower levels of *UPF3B* (normalized to RPL19.) Patient cells harbor a nonsense mutation that subjects UPF3B mRNA to NMD. B-D. NMD substrates are more highly expressed in both control and patient iPSCs relative to fibroblasts, particularly in patient iPSCs, suggesting NMD is weaker after reprogramming.
Figure 3.7 Exogenous UPF3B rescues the NMD deficiency in patient iPSCs. A-B. Exogenous expression of UPF3B from a lentiviral vector rescue UPF3B levels to about 3 times that of control iPSCs. Expression of NMD factors UPF1 and UPF2 are unaffected by UPF3B rescue. NMD substrate upregulation was reversed with UPF3B rescue. C. Upregulation of classical NMD substrates is reversed with UPF3B rescue, while expression of UPF3B-independent substrates is unaffected. Expression levels normalized to RPL19.
Figure 3.8 Control and patient iPSC express pluripotency factors. A-B. qPCR analysis revealed massive upregulation of the pluripotency factors, *NANOG*, *OCT4*, and *SOX2* in both control and patient iPSC, indicating successful reprogramming. B. Control and patient iPSCs stain positive in immunohistochemistry for OCT4 and NANOG.
MATERIAL AND METHODS

Fibroblast Cell Culture

Control and patient fibroblasts were obtained in accordance with ESCRO regulations. Family 1 fibroblasts were cultured in HAMS F-10 with GlutaMAX media, 10% FBS and 1% Pen/Strep. Family 2 fibroblasts were cultured in RPMI media and 10% FBS. Family 3 fibroblasts were cultured in DMEM media, 10% FBS, 1% L-glutamine and 1% Pen/Strep. Male human dermal fibroblasts for CRISPR knockout were cultured in DMEM:F12 and 15% FBS. All cells were cultured at 37°C and 5% CO2.

Reprogramming

Family 1 fibroblasts were reprogrammed through the Sanford-Burnham Stem Cell Core. The episomal pCXLE-Oct4/shP53, pCXLE-Sox2/Klf4, and pCXLE-LMyc/Lin28 vectors were introduced by electroporation. In the second round of reprogramming, Family 1, 2, 3, and CRISPR-knockout fibroblasts were reprogrammed with retroviral transduction of pMXs-hOCT4, pMXs-hSOX2, pMXs-hKLF4, and pMXs-hC-MYC.

iPSC Culture

iPSCS were originally grown on irradiated mouse embryonic feeder cells in WiCell media (DMEM:F12 with GlutaMAX, 20% KnockOut Serum Replacement, 1% Non-Essential Amino Acids, 0.1mM 2-mercapotethanol and 12ng/ml bFGF. Cells were passaged manually. After 2-3 passages, iPSCs were adapted to feeder-free
conditions on Geltrex matrix (Life Technologies) and Essential 8 media (Life Technologies.) Media was changed daily.

**CRISPR Validation**

CRISPR validation was performed by T7EI assay according to manufacturer’s protocol (New England BioLabs.) Gel-shift mobility assay was performed as described in (Ota *et al*, 2013; Chen *et al*, 2012).

**qPCR Gene Expression Analysis**

RNA isolation was performed as per the Trizol Reagent protocol. RNA was reverse-transcribed with iScript Transcriptase (Bio-Rad), and qPCR analysis conducted with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) according to protocol.

**Immunohistochemistry**

Cells were fixed for 20 minutes in 4% paraformaldehyde, washed 3 time with PBSm and blocked in 10% donkey serum, 1% BSA, and 0.1% Triton-X for 1 hour at room temperature. Goat anti-OCT4 and rabbit anti-NANOG were incubated at 1:100 and 1:500 in 10% blocking buffer overnight at 4C. Cells were washed with PBS and incubated with donkey anti-goat and anti-rabbit secondary antibodies in 10% blocking buffer for 1 hour at room temperature.
ACKNOWLEDGEMENTS

Chapter 3, in full, is being prepared for submission. The dissertation author is the primary investigator and co-first author on this publication.

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CHAPTER 4: THE ROLE OF UPF3B IN PLURIPOTENCY AND DIFFERENTIATION IN IPSCS
INTRODUCTION

Factors in Pluripotency and Differentiation

Epigenetic and Transcriptional Regulation

Regulation of pluripotency occurs at multiple levels—epigenetic, transcriptional, post-transcriptional, and translational. Recent evidence demonstrates that epigenetic mechanisms are particularly important for pluripotency, including nucleosome and chromatin remodeling, histone modifications, and methylation/acetylation (Rais et al., 2013; Singhal et al., 2010; Ang et al., 2011; Mansour et al., 2012; Sridharan et al., 2013; Costa et al., 2013).

The core transcription factors controlling pluripotency, NANOG, OCT4, and SOX2, co-occupy over 1000 target genes; they activate genes promoting pluripotency and repress genes directing developmental processes. Numerous other factors, including MDB3 and WDR5, participate in the intricate networks controlling the transcription of genes that are required for or promote pluripotency (Rais et al., 2013; Ang et al., 2011).

Post-transcriptional Regulation

Once transcribed, pluripotency genes give rise to RNAs that are subject to regulation through RNA-binding proteins that modify and ultimately affect their expression. Many RNAs that encode pluripotency factors exist as several splice variants/isoforms, only a small portion of which are active in the pluripotent state. For example, the SON, SRSF2, and MBNL1/2 splicing factors play critical roles in
regulating the expression of variants that are active in and promote pluripotency (Lu et al, 2013, 2014; Han et al, 2013). Similarly, factors such as FIP1 are essential to maintain the proper polyadenylation profiles of various pluripotency mRNAs (Lackford 2014 EMBO). Yet another source of post-transcriptional regulation lies in RNA modification, whereby RNA editing enzymes including TUTases, Adar, and Mettl3/13 modify their target transcripts in such a way as either promote or inhibit differentiation and self-renewal (Hagan et al, 2009; Osenberg et al, 2010; Wang et al, 2014). Downstream of these, factors controlling the localization, and stability of transcripts, both directly and indirectly impact when and to what level they are translated into functional proteins. Numerous microRNAs, such the central and well-known Let-7, and factors involved in microRNA biogenesis and function have been shown to repress expression of genes that promote differentiation of stem cells (Chang et al, 2012; Rybak et al, 2009; Loedige et al, 2013). Other factors, such as PUM1 and BRF1, inhibit expression of genes that promote pluripotency (Tan & Elowitz, 2014; Leeb et al, 2014).

Evidence for a Role of UPF3B in Pluripotency and Differentiation

Despite the plethora of literature documenting various post-transcriptional mechanisms regulating pluripotency, none yet have described the role of NMD in this process. Supporting the idea that NMD plays a role in pluripotency and differentiation, data mining of RNA-seq database of 452 embryonic or induced pluripotent stem and 252 differentiated cell lines (Müller et al, 2011) by the Jeanne Loring and Louise Laurent laboratory revealed that three NMD factors, RNPS1,
MAGOH, and most interestingly, UPF3B are significantly upregulated in pluripotent lines, suggesting NMD functions in stemness (Lou et al., submitted).

Recently, my laboratory has found that NMD plays a critical role in maintaining stemness in multipotent cells and that NMD must be downregulated to permit neural differentiation. We found that NMD promotes stemness by selectively degrading mRNAs encoding factors promoting neural differentiation and inhibiting proliferation (Lou et al., 2014a). Focusing on the role of NMD in neural differentiation, we found that maintenance of UPF1 by exogenous expression effectively blocks P19 differentiation down the neural lineage, with persisting expression of pluripotency markers, OCT4 and NANOG.

Furthermore, a project scientist in the laboratory, Dr. Chih-Hong Lou, found recently and in line with its role in pluripotency that NMD directs germ layer specification and differentiation (Lou et al., manuscript submitted). In embryoid body and directed differentiation of H9 embryonic stem cells into the three germ layers, NMD factor expression profiles vary depending on germ layer specification. To focus on the “core” NMD factors, he found that UPF1, UPF2, and UPF3B all increase in expression in ectoderm, while in mesoderm only UPF1 and UPF3B increase. In endoderm, UPF1, UPF2, and UPF3B all decrease in expression. Rescue experiments in which these factors were either exogenously expressed or depleted to counter the natural expression changes occurring in differentiation were sufficient to effectively block effective differentiation into either endoderm or mesoderm.
This data in part corroborates the cellular and molecular data from our \textit{Upf3b}-null mice, which exhibit a predisposition to differentiation down the neural lineage (Lou \textit{et al}, 2014b). Taken together with our data on neural deficits in \textit{Upf3b}-null mice and \textit{UPF3B} human intellectual disability patients, this leads to the hypothesis that the UPF3-dependent branch of NMD promotes neural stem cell self-renewal by degrading transcripts that promote differentiation and inhibit proliferation and that it must be downregulated for proper neural development and function. In this chapter, I examine the role of UPF3B in pluripotency and differentiation.
As I cultured the iPSCs, I observed that the patient cells exhibited distinct growth characteristics relative to control iPSCs (Table 4.1). Particularly in feeder growth condition, patient cells more frequently spontaneously differentiated, took longer to adhere after passaging, and reached confluence post-passaging several days after controls passaged at the same time. Their tendency to differentiate corroborated the finding of my colleague, Dr. Chih-Hong Lou, that NMD is essential to maintaining pluripotency and that NMD-deficient cells are prone to differentiation. (Lou et al, 2014b) Adhesion and proliferation defects could also contribute to a reduced ability to maintain pluripotency.

Within the scope of neurodevelopment, a tendency to differentiate could impair proper neurodevelopmental timing as stem cells prematurely differentiate. Furthermore, adhesion and proliferation are essential for proper self-renewal and retention/migration out of stem cell niches during neurodevelopment. Not surprisingly then, adhesion defects have been implicated in a number of neurodevelopmental disorders, including intellectual disability and autism.

Based on this, I decided to focus my efforts on examining the role of UPF3B in pluripotency and differentiation, while also probing possible adhesion and proliferation defects as factors indicative of reduced ability to maintain pluripotency.
**Table 4.1 Patient iPSCs exhibit aberrant growth characteristics.** Patient iPSCs noticeably were prone to differentiate, particularly when cultured on MEFs. Compared to control iPSCs passaged in the same manner and at the same time, patient cells took far longer to attach and reach confluency post-passage.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Potential Explanation</th>
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<tr>
<td>Prone to differentiate</td>
<td>Reduced expression of genes promoting pluripotency</td>
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<tr>
<td>Slow to adhere</td>
<td>Reduced expression of adhesion molecules</td>
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<tr>
<td>Slower to reach confluency</td>
<td>Proliferation defect or cell death</td>
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RESULTS

Pluripotency

Alkaline Phosphatase (AP) Staining:

Based on immunohistochemistry, OCT4 and NANOG expression was not apparently different between control and patient cells (Figure 3.8). In contrast, patient iPSCs had reduced alkaline phosphatase (AP) staining (Figure 4.1). This latter result corroborated the observation that patient iPSCs were more prone to spontaneously differentiate. Consistently across patient clones, AP staining was more sparse and uneven relative to control cells, which exhibited a strong and uniform staining. This suggests that patient cells are less able to maintain pluripotency and potentially within the population, patient cells stochastically lose pluripotency.

Pluripotency Factor Expression:

Because the staining approaches undertaken were not amenable to quantification, I examined pluripotency factor expression at the RNA level. Considering their tendency to differentiate, patient iPSCs paradoxically expressed higher levels of pluripotency factors, *NANOG*, *OCT4*, and *SOX2* (Figure 4.2). As the patient cells appeared to be more differentiated, this seemingly contradictory data in fact may demonstrate a general misregulation of pluripotency factors in patient cells. This inconsistency and general confusion in patient cells may in turn promote the spontaneous differentiation observed in culture. Rescue of patient cells with
exogenously expressed UPF3B reversed the upregulation of pluripotency factors, implicating the role of UPF3B in this phenotype.

*Pluripotency in minimal media:*

To further assess this phenotype, I assessed pluripotency factor expression in response to bFGF dose. The rationale behind this experiment was that limiting this essential growth factor would impede the ability of the already differentiation-prone patient cells to maintain pluripotency. Using Essential 6 media, which lacks TGFB1 and bFGF factors provided in the full Essential 8 media, I dosed bFGF at 0ng, 1ng, and 5ng (which comprise the low range of typical bFGF in other medias) and 100ng, the concentration at which bFGF is supplied in Essential 8 media.

Despite the low bFGF does, both control and patient cells maintained normal pluripotent stem cell morphology for the first passage. Upon continued passaging in the low bFGF media, both control and patient cells then adopted a highly differentiated morphology. OCT4 and NANOG respond in a dose-dependent manner to bFGF concentration in both control and patient cells, while c-MYC expression is unchanged at low bFGF concentrations and increases only at the 100ng dose. Sox2, however, exhibits relatively consistent expression across bFGF concentrations, except at the 5ng dose in patient cells. UPF3B expression in control cells somewhat surprisingly decreases with increasing bFGF dose and is most highly expressed at the 0ng bFGF concentration. Given my hypothesis that UPF3B is needed to maintain pluripotency, the cells may upregulate UPF3B to counter the differentiation-inducing low bFGF media (Figure 4.3).
Interestingly, at all bFGF concentrations, \textit{OCT4}, \textit{c-MYC}, and \textit{NANOG} were upregulated in patient iPSCs. Surprisingly, except at the 5ng dose, Sox2 is downregulated in patient cells relative to control cells. Examination of pluripotency gene expression between Essential 6 and Essential 8 media revealed slight perturbations, which presumably arise from the differences in media composition. Of note, \textit{OCT4} was dramatically upregulated in patient cells grown in E6, while control cells were unchanged in \textit{OCT4} expression levels. \textit{SOX2}, on the other hands, was highly upregulated in controls cells, which explains why \textit{SOX2} then in comparison appeared downregulated in the patient cells when grown in E6 media. These data suggest that as a general principle, pluripotency factor expression depends on the concentration and type of growth factors/signaling molecules present in the media. The UPF3B deficiency affects how the cells then respond to these changes in external cues, with the most dramatic changes in \textit{OCT4}, and \textit{SOX2} expression.
Figure 4.1 Patient cells exhibit sparse and uneven alkaline phosphatase staining. Control and patient cells were seeded for alkaline phosphatase staining under the same conditions and at the same time. Staining is uniform and strong in control colonies but patchy and weak in patient cells. Growth rate differences are apparent after 4 days of culture in the identical conditions.
Figure 4.2 Pluripotency factors, NANOG and SOX2, are upregulated in patient cells relative to control. A. NANOG and SOX2 are upregulated in patient cells, while c-MYC and OCT4 are expressed at similar levels in control and patient cells. B. Rescue of patient cells reverses upregulation, implicating UPF3B in pluripotency factor misregulation in patient cells. Expression levels normalized to RPL19. Average of 3 control clones and 3 patient clones.
Figure 4.3 Control and patient iPSC exhibit a partial dose-dependent response to bFGF. A and D. c-MYC and SOX2 expression in both control and patient cells do not exhibit a dose response to bFGF, while (B-C) NANOG and OCT4 demonstrate clear dose dependency. E. UPF3B expression decreases with increased bFGF dose. F. Pluripotency factor expression is in most cases unchanged in both control and patient cells when cultured in E6 media (used for bFGF dosing.) Expression levels normalized to RPL19. Average of 2 control clones and 1 patient clone.
Proliferation

Proliferation and self-renewal are a defining characteristic of pluripotent stem cells. As seen in the alkaline phosphatase staining, I found that the control and patient cells displayed a clear difference in growth pattern. Though both control and patient cells were seeded at similar densities and grown for an equal number of days, the patient cells are far less confluent relative to control. This potential proliferative defect may account for the patient cells’ reduced capacity to maintain pluripotency. This observation of reduced proliferative activity was validated by an EdU-incorporation assay that quantifies cell proliferation by measuring incorporation of an uridine analog that can be labeled with a fluorescent probe. The data reveal as expected that patient iPSCs exhibit reduced proliferation relative to control (Figure 4.4).

Cell Cycle and Apoptosis

To probe the dramatically different growth characteristics of the patient iPSCs more deeply, I assayed the cells for differences in cell cycle and apoptosis. Propidium iodide staining revealed subtle differences in cell cycle, as patient cells had fewer cells in G1 but more in G2 relative to control. The proportion of cells in S phase was unchanged. This suggests that patient cells tend to more rapidly exit G1 but are blocked at G2, impeding their entry into mitosis and thus reducing their proliferative ability (Figure 4.5). There was no change in apoptosis as assayed by PI staining. To validate this, cells were stained for and quantified for early apoptosis surface marker, Annexin V by flow cytometry. Annexin V staining confirmed previous result and
showed no changed in apoptotic activity between control and patient cells. (Data not shown.)
Figure 4.4 Patient cells exhibit a proliferation defect relative to control cells. EdU-incorporation in actively dividing cells is significantly reduced in patient cells (quantified by Click-It staining.) Cells were incubated for 30min with EdU and stained with a Click-It probe that binds incorporated EdU. EdU positive cells were counted manually across several colonies and averaged. Average of 3 control clones and 3 patient clones.
Figure 4.5 Patient cells accumulate at G2-M compared to control cells. PI staining revealed that patient cells have a higher proportion of cells in G2-M relative to control cells, while the proportion of S phase cells is slightly reduced. This may indicate misregulation of the cell cycle or a delay in cell division, which is consistent with the cell proliferation defect observed in patient cells. Average of 3 control clones and 3 patient clones.
Differentiation

*Embryoid Body (EB) Differentiation:*

To assess the differentiation capacity of the iPSCs, I generated embryoid bodies using two approaches. In the first approach – the hanging drop method – droplets of uniform iPSC density are suspended from the culture dish lid, which is thought to generate more uniform embryoid bodies (Shevde *et al* 2013). Uniformity of embryoid bodies minimizes structural differences from biasing germ layer differentiation. The second approach is to generate embryoid bodies in low attachment dishes, which is a more efficient method than the hanging drop method but gives rise to embryoid of unequal size and morphology.

While control iPSCs formed embryoid bodies without difficulty, patient iPSCs did not survive the handling to generate embryoid bodies without the addition of rock inhibitor, particularly with the hanging drop approach. Under the same seeding conditions, the number of EBs formed was 10-fold lower in patient cells relative to control.

Gene expression in embryoid bodies generated in the low attachment plate approach was inconsistent and erratic. Results described here after refer those obtained with embryoid bodies generated from hanging drops. In both control and patient embryoid bodies, pluripotency marker expression dropped massively by Day 14 (Figure 4.6, left column). Consistent with the idea that UPF3B promotes pluripotency and must be downregulated to permit differentiation, UPF3B expression dropped throughout EB differentiation (Figure 4.6A).
Analysis of germ layer markers demonstrated that the embryoid bodies differentiated into cells belonging to the three lineages. In control iPSC, ectoderm markers $PAX6$ and $SOX1$ were most abundant (Figure 4.7A,C), while markers for mesoderm, $NODAL$ and $BRACHYURY$, and endoderm, $SOX17$, were expressed at modest levels (Figure 4.7E,G,I). In patient embryoid bodies, however, $SOX17$ was most abundant, followed by $PAX6$ and $SOX1$. The level of induction of $SOX17$ was 10-fold higher in patient embryoid bodies.
Figure 4.6 Pluripotency markers decrease during embryoid body (EB) differentiation in both patient and control cells. Embryoid bodies were generated using the hanging drop (HD) and low attachment plate (LA) approaches. In both control and patient cells, pluripotency markers drop over the course of EB differentiation. A-B. Of note, UPF3B expression drops in control cells, supporting the idea that its promotes pluripotency. C-J. Overall, C-MYC, NANOG, OCT4, and SOX2 decrease in expression during EB differentiation, consistent with a differentiated state. Expression levels set to control cells at Day 1, and normalized to RPL19. 1 control clone and 1 patient clone.
Figure 4.7 Germ layer markers increase during embryoid body (EB) differentiation in both patient and control cells. Embryoid bodies were generated using the hanging drop (HD) and low attachment plate (LA) approaches. In both control and patient cells, germ layer markers increase during the course of EB differentiation. A-D Ectoderm markers *PAX6* and *SOX1* increase, particular in control cells. E-F Mesoderm marker, *BRACHYURY*, increases in both HD and LA EBs by Day 14. G-H. Mesoderm and early endoderm marker, *NODAL*, varies in expression through EB differentiation while I-J. Endoderm marker, *SOX17*, is dramatically induced in patient cells derived using both HD and LA approaches. Expression levels set to control cells at Day 1, and normalized to RPL19.
Endoderm Differentiation

Based on the embryoid body data, which indicates patient iPSC propensity to differentiate down the endodermal lineage, I differentiated both control and patient lines using Activin A/BMP4 and assessed for SOX17 expression. While there was negligible upregulation of SOX17 in non-differentiated patient cells, following complete endoderm differentiation, SOX17 was dramatically higher in patient cells, indicating a propensity to differentiate down the endoderm lineage relative to control cells. It is induced roughly 3500 fold from the starting iPSC, while control cells exhibit a more modest 900 fold induction. Validating the loss of pluripotency factor expression, SOX2 expression dropped to near undetectable levels in control cells and slightly higher levels in patient cells. UPF3B levels dropped to about 50% of starting in control cells (Figure 4.8) in line with my colleague Dr. Chih-Hong Lou’s analysis of endoderm differentiated H9 cells (Lou et al., submitted).

To ascertain that the observed effect are in fact attributable to UPF3B, I exogenously expressed UPF3B in patient cells prior to endoderm differentiation and assessed the affect of introducing UPF3B back into patient cells on endodermal differentiation. With UPF3B expression a few fold increased over patient and near control levels, the data revealed a reversal of the patient endoderm differentiation phenotype. Both patient and rescue cells demonstrated an induction of SOX17 during differentiation. However, cells with UPF3B rescue exhibited reduced SOX17 expression relative to unrescued patient cells, suggesting that UPF3B repression indeed promotes endodermal differentiation (Figure 4.9).
Figure 4.8 Control and patient iPSCs differentiate into endoderm. A. *SOX17* is dramatically induced by endoderm differentiation with Activin A/BMP treatment, especially in patient cells. B. Pluripotency marker, *SOX2*, drops to near undetectable levels indicating loss of pluripotency. C. *UPF3B* expression drops significantly in control cells, consistent with the idea that tUPF3B repression is required for endoderm differentiation. Expression levels set to control cells at Day 1, pre-differentiation and normalized to RPL19. Average of 3 control and 3 patient clones.
Figure 4.9 Patient iPSCs with UPF3B rescue demonstrate a reversal in Sox17 expression during endoderm differentiation. A. UPF3B rescue of patient cells reduces SOX17 expression relative to control cells. B. SOX2 expression drops during endoderm differentiation, indicating loss of pluripotency. C-D. UPF3B expression is rescued to about 50% of control UPF3B levels. E. SOX17 expression in differentiated patient with UPF3B rescue cells is higher than control cells but reduced from patient cells transduced with empty vector, demonstrating the role of UPF3B depletion in promoting endoderm differentiation. Data shown from one patient line and corresponding rescue line. Similar results were obtained in a second patient line (data not shown.)
Cortical Neural Progenitor Differentiation

To assess the neural differentiation phenotype of these patient cells, I directly differentiated control and patient fibroblasts into neural stem cells using SOX2 alone (Ring et al., 2012). Patient cells exhibited morphological changes consistent with that of neural progenitor reprogramming earlier than control cells (Figure 4.10). At the molecular level, patient cells expressed higher levels of early neural marker, NESTIN, suggesting that patient cells are predisposed to or more rapidly differentiate down the neural lineage (Figure 4.11). These changes cannot be directly attributed to UPF3B as reprogramming efficiency depends on age of fibroblasts being reprogrammed. NESTIN upregulation in addition to the more rapid conversion however, suggests that UPF3B contributes to the neural conversion process.

Because the cortex is responsible for learning and memory, which is impaired in all UPF3B patients, I differentiated control and patient iPSCs into cortical progenitors that eventually give rise to all neurons of all cortical layers (Shi et al., 2012a, 2012b). Both SOX1 and PAX6 were upregulated in control and patient cells by Day 12 of differentiation, indicating successful reprogramming (Figure 4.12). Comparing control and patient NPCs, patient cells at the iPSC stage expressed higher levels of both SOX1 and PAX6, which would suggest a predisposition to differentiate down the neural lineage. While this difference was maintained in NPCs, the magnitude of upregulation was much reduced (Figure 4.12). The data generated here is still preliminary; it is possible that UPF3B functions during particular stages of cortical differentiation and not at the particular timepoints sampled. Further study is
needed to fully evaluate the role of UPF3B in neural differentiation in general and particularly in cortical neuron differentiation to examine its roles in these neurodevelopmental disorders.
Figure 4.10 Patient fibroblasts more rapidly differentiated into neural progenitor cells. In direct conversion of fibroblasts to neural progenitor cells, patient cells more rapidly acquired morphological changes consistent with that of neural progenitors and neurospheres. (Day 7 panels, at top) Control cells eventually adopted morphological changes. By Day 14 (bottom panels,) patient cells exhibited a more uniform and robustly proliferating neural progenitor cell population.
Figure 12.11 Reprogrammed neural progenitor cells (NPCs) from patient cells express higher levels of NESTIN. A. In control and patient fibroblasts directly converted into neural progenitor cells, patient NPCs express higher levels of the early neural marker, NESTIN. B. UPF3B expression increases during neural progenitor conversion. Expression levels set to control cells at Day 1, pre-differentiation and normalized to RPL19.
Figure 4.12 Patient iPSCs differentiated in cortical neuron progenitors express higher levels of NPC markers, PAX6 and SOX1. A-B. PAX6 and SOX1 levels were higher in patient-derived NPCs. C. UPF3B expression increased during NPC differentiation. Expression levels set to control cells at Day1, pre-differentiation and normalized to RPL19. Average of 3 control clones and 3 patient clones.
**Genome-Wide Sequencing**

RNA-Seq analysis revealed that roughly 10% of the genome was upregulated, 10% downregulated, and 80% unchanged in patient cells relative to control (Figure 4.13). I was most interested in transcripts that were upregulated in patient cells as these are likely to be NMD targets. Gene ontology analysis categorized the upregulated transcripts in functional categories that might be involved in regulation of pluripotency and differentiation, including “mRNA transcription regulation,” “cell proliferation/differentiation,” and “cell cycle control.” Downregulated transcripts, which could be affected by NMD indirectly, largely function in various metabolic, biosynthetic, and protein modification processes (Table 4.2).

In line with my hypothesis that UPF3B functions in stem cell self-renewal/differentiation and neurodevelopment, many differentially regulated transcripts are involved in pluripotency/differentiation, adhesion, proliferation as well as neurodevelopmental disorders and neural development/function. To highlight a few functional categories: NODAL and BMP2/4/7 belong to the TGF-beta superfamily and function in early development, particularly in germ layer specification and proper organization of the developing tissues/structures. OTX2 and SOX21 are transcription factors that function in brain development. OTX2, a homeodomain-containing-transcription factor, directs early brain development by dictating midbrain and forebrain patterning. SOX21 belongs to the Sox family transcription factors and promotes differentiation of neural stem cells. As implied by the name, autism susceptibility candidate 2 (AUTS2,) is implicated in autism, intellectual disability, and
developmental delay, all of occur in UPF3B patients. SYNGAP1 is critical to synaptic function and when mutated causes intellectual disability. Many of these transcripts also contain features known to induce NMD (Table 4.3). Except for OTX2, all transcripts were significantly upregulated as assessed by qPCR (Figure 4.14). This upregulation was reversed in patient cells with UPF3B rescue, demonstrating UPF3B-dependence (Figure 4.14).
Figure 4.13 RNA-Seq analysis reveals roughly 20% of the transcriptome is differentially expressed in patient cells relative to control cells. Of 11000 gene transcripts detected, approximately 1000 are upregulated, 1100 downregulated, and 9000 unchanged. Statistical significance was set at 1.5 fold change and q<0.05. Average of 3 control and 3 patient clones.
### Table 4.2 Functional categories of differentially expressed transcripts.
Differentially expressed transcripts fall into functional categories involved in pluripotency/differentiation and neurodevelopment.

<table>
<thead>
<tr>
<th>Phenotype/Function</th>
<th>Candidate misregulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluripotency/differentiation defect</td>
<td><strong>AXIN2, BMP2/4/7, FOXD3, GDF3, SOX21</strong></td>
</tr>
<tr>
<td>Adhesion defect</td>
<td><strong>CADM1, CTGF, CYR61, JAM2, LAMA5</strong></td>
</tr>
<tr>
<td>Growth defect</td>
<td><strong>CASP2, CDK6/10, DAPK3, ING3</strong></td>
</tr>
<tr>
<td>Neurodevelopmental disorders</td>
<td><strong>AUTS2, CACNA1H, NIPAL1, SYNGAP1</strong></td>
</tr>
<tr>
<td>Neural development/function</td>
<td><strong>ADCY8, ATP1B2, CAPRIN2, CBLN1, CHAC1, DCLK1, EGR1, ENPP2</strong></td>
</tr>
</tbody>
</table>
### Table 4.3 NMD-inducing features of differentially expressed transcripts.

Several transcripts functioning in pluripotency/differentiation and neurodevelopment contain NMD-inducing features.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NMD Inducing Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AXIN2</em></td>
<td>5’ uORF, 1.4kb 3’ UTR</td>
</tr>
<tr>
<td><em>BMP2</em></td>
<td>5’ uORF, 1.2kb 3’ UTR</td>
</tr>
<tr>
<td><em>BMP4</em></td>
<td>5’ uORF</td>
</tr>
<tr>
<td><em>BMP7</em></td>
<td>2.2kb 3’ UTR</td>
</tr>
<tr>
<td><em>NODAL</em></td>
<td>none</td>
</tr>
<tr>
<td><em>OTX2</em></td>
<td>1.8kb 3’ UTR</td>
</tr>
<tr>
<td><em>SOX21</em></td>
<td>1.6kb 3’ UTR</td>
</tr>
<tr>
<td><em>AUTS2</em></td>
<td>5’ uORF, 1.5kb 3’ UTR; 2 transcripts predicted NMD targets (intron in 3’ UTR)</td>
</tr>
<tr>
<td><em>SYNGAP1</em></td>
<td>1.8kb 3’ UTR; 1 transcripts predicted NMD target (intron in 3’ UTR)</td>
</tr>
</tbody>
</table>
Figure 4.14 Differentially expressed transcripts identified in RNA-Seq analysis are upregulated in patient cells. A. Differentially expressed transcripts are highly upregulated in patient cells. *OTX2* is not significantly upregulated. B. UPF3B rescue reverses upregulation of most transcripts, demonstrating UPF3B-dependency. *NODAL* expression does not change with UPF3B rescue, suggesting it is not an NMD substrate. Of all transcripts assayed here, it is the only without an NMD-inducing features. Average of 3 control and 3 patient clones. Rescue is one patient lines, 6 replicates.
MATERIALS AND METHODS

UPF3B Rescue

UPF3B was exogenously expressed through lentiviral transduction of a UPF3B overexpression construct driven by the CAG promoter. Cells were transduced with 4ug/ml polybrene three days prior to cell harvest or 2 days prior for differentiation experiments.

Proliferation Assay

Proliferation was assessed with EdU incorporation. Following a 30 minute incubation with EdU, cells were fixed in 4% paraformaldehyde and stained with a EdU fluorescent Click-It probe that binds incorporated EdU. Cells incorporating EdU were counted manually and averages were taken across several colonies. (assay kit available from ThermoScientific.)

Apoptosis Assay

Apoptosis was assayed by expression of early apoptotic marker, Annexin V and FITC staining. (assay kit available from Sigma) Cells were fixed and stained with Annexin V and Flow cytometry was performed by the VA Hospital Flow Cytometry Core.

Cell Cycle Analysis

Cell colonies were broken down into single cell suspension and fixed dropwise in ice cold 70% ethananol. Propidium iodide (PI) staining and flow cytometry to quantify DNA content was performed by the VA Hospital Flow Cytometry.
**Embryoid Body Differentiation**

Cell colonies were broken down into single cell suspension or small clumps in the presence of 10uM rock inhibitor in E6 media. For the hanging drop approach, cell suspensions droplets were pipetted onto the lid of a 150mm tissue culture dish lid, which was then inverted over the dish bottom containing a media. For the low attachment plate approach, cell colonies were broken down into small clumps and transferred to low attachment plates. With both approaches, embryoid bodies formed overnight and were transferred after 2 days to ultra-low attachment dishes. Media was changed every other day after collecting EBs by gravity. EBs were harvested for RNA isolation with Trizol at designated timepoints.

**Endoderm Differentiation**

Cells were seeded in 12-well plates in the presence of 10uM rock inhibitor the day prior to differentiation as small clumps at high density (~70% confluency). Media was changed to differentiation media consisting of 1:1 RPMI/DMEM:F12, insulin, and 1X B-27 supplement to which the following factors were added fresh each day: 100ng/ml Activin A, 10ng/ml BMP4, 25n/ml bFGF, 10uM Ly, and 3um CHIR. CHIR was withdrawn after Day 1 of differentiation. Differentiation media was changed daily for 3 days. Cells were harvested for RNA with Trizol.

**Direct Neural Progenitor Cell Conversion**

Early passage control and patient fibroblasts were directly converted into neural progenitor cells as per Ring *et al*, 2012.
Cortical Progenitor Differentiation

Control and patient iPSCs were differentiated into cortical neuron progenitors as per Shi et al, 2012.

RNA-Seq Analysis

RNA samples were isolated according to Trizol protocol and re-precipitated to improve purity and quality. Library preparation and sequencing was performed by the IGM Sequencing Core. Analysis was performed using TopHat and Cufflinks analysis packages.
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Chapter 4, in full, is being prepared for submission. The dissertation author is the primary investigator and co-first author on this publication.

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CHAPTER 5: DISCUSSION
DISCUSSION

In summary, I have identified a microRNA regulatory circuit in which the neurally enriched microRNAs, miR-132 and miR-9/-124/-128, repress the UPF2 and UPF3B branches of NMD, respectively. To examine the role of the UPF3B branch of NMD in neurodevelopment, I derived iPSCs and NPCs from patients with \textit{UPF3B}-null mutations. Using these \textit{UPF3B}-deficient iPSCs and NPCs, I obtained substantial evidence that UPF3B promotes pluripotency, while depletion or absence of UPF3B promotes differentiation.

\textbf{UPF3B and the microRNAs, miR-9, -124, and -128, Form a Circuit that Serves as a Developmental Switch}

The neurally expressed microRNAs, miRs-9, -124, and -128, are known to play important roles in regulating brain development (Li & Jin, 2010; Fineberg \textit{et al}, 2009). My work suggests that the ability of these microRNAs to influence brain development may stem, in part, through their ability to regulate NMD. The reciprocal expression patterns of UPF3B and these microRNAs during embryonic brain development support this idea; decrease in UPF3B expression is concomitant with increase in miR-9, -124, and -128 expression during embryonic brain development (Figure 2.1). These data support the idea that microRNA-mediated repression of NMD either triggers neural differentiation or that sustained NMD repression via microRNAs is necessary for the proper execution of neurodevelopment programs.
Evidence from patient-derived induced pluripotent stem cells lacking UPF3B corroborates the idea that UPF3B must be downregulated for neural differentiation. This is also supported by previous studies showing that UPF1 is downregulated during neural differentiation and that this promotes neural differentiation (Bruno et al., 2011; Lou et al., 2014). Using iPSCs generated from patients with UPF3B-null mutations, I found that UPF3B promotes pluripotency. Patient iPSCs lacking UPF3B were predisposed to differentiate into neural progenitor cells, suggesting that the absence of UPF3B in these patients prompts premature or accelerated neural differentiation.

Considering the feedback mechanism described in Chapter 2 by which UPF3B and UPF3B-regulatory microRNAs (miR-9, -124, and -128) mutually repress one another, it is possible that UPF3B-deficient patients express higher levels of miR-9, -124, and -128, in which case the loss of UPF3B is doubly detrimental. Firstly, NMD function is impaired by loss of UPF3B function, causing misregulation of NMD substrates; a subset of the upregulated transcripts in patient iPSCs play important roles in neural development and function. Secondly, loss of UPF3B-mediated repression of these microRNAs would be predicted to elicit more robust downregulation of miR-9, -124- and -128 direct target mRNAs, several of which have been shown to be critical for neural development. In particular, these three microRNAs target a battery of transcripts that promote self-renewal/proliferation, inhibit neural differentiation, or promote migration during corticogenesis (Volvert et al., 2012; Lang & Shi, 2012). For example, miR-9 promotes neural stem cell proliferation and self-renewal by targeting FOXG1 (implicated in Rett Syndrome), REST, and TLX (Shibata et al., 2008; Packer
miR-124 induces neural differentiation by downregulating transcripts that otherwise inhibit this process, including PTBP1, SOX9, and SCP1 (Makeyev et al., 2007; Cheng et al., 2009; Visvanathan et al., 2007). miR-128, which is expressed in more mature neurons, supports the proper migration of maturing NSCs to and within the developing cortex by targeting mRNAs encoding migration-inhibitory proteins, including PHF6 (implicated in intellectual disability), DCX, and Reelin (Franzoni et al., 2015; Evangelisti et al., 2009). It is also known to be misregulated in autism spectrum disorders (Abu-Elneel et al., 2008).

These data support a model in which the neurodevelopmental defects in UPF3B patients are caused not only by direct effects of UPF3B impairment but also by the resultant upregulation of miR-9, -124, and -128 (Figure 5.1). The cortical neural progenitors I generated from the patient iPSCs provide an opportunity to test aspects of this model examining whether 1) expression of miR-9, -124, and -128 is dysregulated in these NPCs as they differentiate, and 2) miR-9/124/128 downstream targets involved in corticogenesis and neural development are dysregulated, such as those listed above.

Examining the Role of Astrocytes in UPF3B-Based Intellectual Disability

Based on the findings described in this dissertation, the regulation of NMD by microRNAs and vice versa may constitute a developmental switch to dictate differentiation or self-renewal decisions in neural cell lineages. In this regard, it is worth noting that miR-124 specifically promotes the neuronal lineage but inhibits astrocyte differentiation (Krichevsky et al., 2006). If miR-124 is upregulated in
UPF3B patients due to loss of the NMD-mediated negative feedback discussed above, proper differentiation of neural stem cells into the full repertoire of neural lineages, including astrocytes, will be impaired or misguided. The upregulation of this microRNA would be predicted to predispose neural stem cells towards neuronal lineages at the expense of astrocytes.

The importance of support cells, such as astrocytes, in neural development is well established; there is increasing evidence in induced pluripotent stem cell models that impaired astrocyte development and function cause neurodevelopmental disorders (Molofsky et al., 2012). In Rett syndrome, disease progression can be reversed or delayed through rescue of MeCP2-deficient astrocytes or culture with normal astrocytes (Maezawa et al., 2009; Lioy et al., 2011). In iPSC and mouse models of intellectual disability and autism spectrum disorders, astrocytes generated from affected cells inhibit proper maturation and synaptic function in neurons (Krencik et al., 2015; Jacobs & Doering, 2010). While proper neural development and neuronal function factor significantly into neurodevelopmental disorders, these recent findings suggest the previously overlooked astrocytes are equally, if not more important, to proper neurodevelopment and function.

The fact that astrocytes are critical for proper neurodevelopment and function, coupled with our finding that loss of UPF3B leads to increased expression of the “anti-astrocyte” microRNA, miR-124, leads me to speculate that the neurodevelopmental defects in patients may in part be due to astrocyte dysregulation caused by miR-124 upregulation. Using the iPSCs generated in this work, it would be particularly
interesting in light of these findings to examine whether proper astrocyte
differentiation and function is impaired by UPF3B-deficiency. If miR-124 is
upregulated in patient NSCs due to the feedback mechanism described in Chapter 2,
astrocyte differentiation would be predicted to be impaired given the inhibitory effect
of miR-124 on gliogenesis (Krichevsky et al, 2006). Should this be the case, the delay
in or restraints on astrocyte development may inhibit neuronal function and signaling,
as demonstrated in previous studies of astrocytes in intellectual disability and autism
spectrum disorders. Because astrocytes are critical not only in function of mature
neurons but also in their development, a possible astrocyte dysfunction may be the
underlying cause to or factor in impaired neuronal differentiation and function. To
that end, the directly converted NPCs generated in this dissertation would be a good
model system to examine in UPF3B-deficient cells whether 1) specification and
commitment to the astrocyte lineage is inhibited, and 2) differentiation and maturation
of astrocyte lineage is impaired.

Examining the role of UPF3B in neuronal differentiation and function

Based on existing evidence, it is likely that the basis for intellectual disability
and other neurodevelopmental disorders cannot be attributed to one cell type alone,
whether astrocytes or neurons. Autism-spectrum disorders constitute a major
proportion of diagnosed neurodevelopmental disorders and occur frequently in
UPF3B-patients. In ASD-affected brains, where defects were observed in all cortical
layers, layers 4 and 5 appeared to be the most severely impacted (Stoner et al, 2014).
Moreover, previous studies have shown that ASD patients often exhibit early brain overgrowth caused by an excess number of neurons (Courchesne et al, 2011).

My data indicated that the population of patient iPSCs differentiated into cortical neuron progenitors expressed higher levels of PAX6 than control iPSCs (Figure 4.12). On interpretation of this data is that patient NPCs express more PAX6 per cell than control NPCs. If so, this suggests that patient iPSCs exhibit accelerated NPC differentiation relative to control iPCSs because UPF3B normally suppresses this differentiation step. Another interpretation of this data is that there are more PAX6-positive patient NPCs than PAX6-positive control NPCs. This could occur if patient NPCs expand more robustly than control NPCs, whether by increased survival or proliferation.

Considering the evidence that ASD patients often display improper neuronal differentiation and cortical layer formation, as well as brain overgrowth, all of which are indicative of impaired corticogenesis, these processes may also be affected in UPF3B patients, particularly in those with autism or autistic features. The cortical neural progenitors generated in this work would be a suitable model system for examining in particular each step of corticogenesis as the protocol generates cortical neurons in a sequential manner that recapitulates in vivo development of the cortex (Shi et al, 2012a, 2012b). The stepwise development of the cortical layers that reflects the timing of corticogenesis in vivo affords the unique ability to dissect the potential role of UPF3B at each developmental stage.
Using these cortical NPCs, further study could examine whether 1) the balance between NSC proliferation/self-renewal and neural specification/commitment is skewed, 2) differentiation of cortical neurons from neural stem cells both in number and timing is affected, and 3) differentiation into and migration of maturing NSCs to particular cortical layers is impaired.

In summary, the work described in this dissertation and the continually emerging data on neurodevelopment and neurodevelopmental disorders form a strong foundation on which to dissect the role of RNA metabolism in intellectual disability, autism, and schizophrenia. This future work has the potential to determine the underlying cause of and potential therapeutic interventions for UPF3B-specific and other neurodevelopmental disorders.

Improved understanding of the mechanisms behind UPF3B in neurodevelopment and function, as well as the cellular models with which to study them, lays the foundation for exploring potential therapies for intellectual disability and related disorders caused by UPF3B or NMD-deficiency. The UPF3B patient-derived neural cells uniquely afford the ability to directly explore and tailor individualized/patient-specific medicine for affected patients. Given the heterogeneity of UPF3B–based intellectual disability and neurodevelopmental disorders, which arises from the unique genetic and epigenetic backgrounds of patients, no one therapy will work for all affected patients. Thus, these patient-derived cells allow us to test in the particular cell type of interest whether a therapeutic intervention is efficacious for the individual patient. For example, considering that miR-9,-124, and -128 are
upregulated with UPF3B depletion, one could to examine whether inhibition of miR-9, -124, and -128 through specific and stable microRNA inhibitors developed for clinical applications can mitigate the neurological defects in UPF3B patients. In a similar vein, RNAi approaches can be used to downregulate the effectors of these neurological phenotypes that are upregulated through loss of UFP3B-mediated repression. With the growing interest in and accessibility of patient-specific induced pluripotent stem cell and gene-based therapeutic technologies, such discoveries and advances may come to fruition in the very near future.
Figure 5.1. Model: UPF3B modulates expression of transcripts involved in neural development through NMD and through repression of miR-9/-124/-128. In UPF3B patients, the loss of UPF3B permits aberrant upregulation of pro-neural differentiation transcripts that are direct NMD targets. Loss of UPF3B-mediated repression of miR-9,-124, and -128 elicits stronger repression of their anti-neural differentiation targets. Together, the misregulation of both NMD and miR-9,-124, and -128 targets impairs proper neurodevelopment in patients.
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