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Role of Deimination for Protein Synthesis in Neuronal Cells

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ROLE OF DEIMINATION FOR PROTEIN SYNTHESIS IN NEURONAL CELLS

By
Di Ding

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ROLE OF DEIMINATION FOR PROTEIN SYNTHESIS IN NEURONAL CELLS

Di Ding

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Deimination refers to conversion of protein-bound arginine into citrulline. In contrast to global hyper-deimination in the brain and eyes, we have found local hypo-deimination in the retinal ganglion cell layer of patients and in a transgenic mouse model of multiple sclerosis. REF, an RNA and export binding protein, was specifically found to undergo loss of deimination in multiple sclerosis, resulting in functional changes in RNA binding. mRNAs for SNARE complex and mitochondrial ATPase complex are enriched by deiminated REF. We confirmed the presence of REF in dendritic site and mitochondrial surface. Only the deiminated form of REF interacts with the eIF4F complex in both cytosolic and mitochondrial surface. Down-regulation of deimination or REF results in decreased neurite outgrowth and reduced the mitochondrial ATP synthase activity compared to the control. Restoration of deimination in the optic nerve results in dramatic improvement in visual function and elongation of neurite length in isolated neurons. Together, these findings support a key role for protein deimination in dendritic protein synthesis and mitochondrial mRNA transport and identify a potential new pathway for early events in the pathogenesis of multiple sclerosis and possibly other neurodegenerative diseases.
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CHAPTER I: Introduction

Posttranslational modifications (PTMs) to proteins are fundamental steps required in the regulation of many cellular processes. In mammalian systems, common PTMs include the phosphorylation, acetylation, and oxidation of amino acid side chains such as that of prolyl, arginyl, lysyl, histidinyl, and cystyl residues; the deamidation of asparaginyl and glutaminyl residues; the racemization and isomerization of aspartyl, asparaginyl and prolyl residues; and the methylation of arginyl and lysyl residues. One important PTM is deimination, in which protein-bound arginine is converted into citrulline (Vossenaar et al., 2003) (Figure 1). The term citrullination is usually interchangeably used for protein deimination, but for consistency in this dissertation, deimination will be used to describe the conversion of protein-bound arginine.

1.1 Deimination and PADs enzymes. Mammalian cells possess several peptidylarginine deiminases (PADs) encoded from PAD1–4 and 6 genes (Vossenaar et al., 2003). Protein deimination has been shown to occur in epidermal, muscle and neuronal tissues; and is catalyzed by a tissue specific PAD depending on the location. PAD1 and 3 catalyze deimination in the skin, PAD2 is the major PAD in the eye and brain, and PAD4 is commonly associated with hematopoietic lineages (Asaga and Ishigami, 2001; Bhattacharya et al., 2006b; Vossenaar et al., 2003). PAD4 has also been implicated in transcriptional repression and is the only PAD with a nuclear localization sequence (Wang et al., 2009). PAD-catalyzed deimination is calcium-dependent, occurs exclusively at protein-bound arginines, and generates ammonia as a byproduct (Vossenaar et
al., 2003). Conversely, free arginines are catalyzed by nitric oxide synthase (NOS) to generate nitric oxide (NO) and free citrulline. As no known tRNA carrier exists for aminoacyl transfer of citrulline, deimination is regarded strictly as a PAD-catalyzed PTM. The enzymatic activities of PADs are typically defined by the conversion rate of benzoylarginine to benzoylcitrulline, as measured from the absorbance at 470nm (Liao et al., 2005; Yoshionari et al., 1994).

Direct observation of deimination has proven elusive due to similarities in both the structure and molecular weight of arginine and citrulline. Early detection attempts relied upon citrulline modification to a functionality that could be recognized by specific antibodies to this modification. In this two step reaction process, protein-bound citrulline was reacted with 2,3-butanedione monoxime and antipyrine in a strongly-acidic environment to generate an antigenic adduct (Senshu et al., 1992). Recently, antibodies such as F95 (Nicholas and Whitaker, 2002) and ab6464 (abcam) have been developed to detect protein-bound, unmodified citrulline; however, the specificity and sensitivity are still uncharacterized. Deimination has been reported in astrocytes (Bhattacharya et al., 2006a), microglia and oligodendrocytes (Asaga and Ishigami, 2001), and Schwann cells (Keilhoff et al., 2008), and it likely occurs in neurons as well. Currently, only a few proteins such as keratin, myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), vimentin, trichohyalin, histones (H2A, H3 and H4), filaggrin, and fibrinogen have been reported to undergo deimination (Enriquez-Algeciras et al., 2011). More specifically, 2',3'-cyclic-nucleotide 3'-phosphodiesterase, MBP and myelin-associated glycoprotein (MAG) have been
shown to undergo deimination in human retinal tissue (Bhattacharya et al., 2006c) and further independent investigation has confirmed their deimination in the brain as well (Wood et al., 2008). Quantitative mass spectrometry has subsequently identified additional proteins that undergo deimination (Grant et al., 2007).

1.2 Deimination studies in Multiple Sclerosis. Multiple sclerosis (MS) is a neurodegenerative disease characterized by recurrent inflammation and the formation of localized demyelinated plaques in the central nervous system (CNS; brain and spinal cord) associated with axonal degeneration (Ziemssen, 2005). MS has an underlying multifactorial etiology (Ziemssen, 2005) and frequently is multiphasic (Frohman et al., 2008) and multifocal (Pittock and Lucchinetti, 2007). MS has heterogeneous manifestations and is often disabling via deficits of sensory, motor, autonomic, and neuro-cognitive function. MS manifests with features of vision loss, extra-ocular muscle movement disorders, paresthesias, loss of sensation, weakness, dysarthria, spasticity, ataxia, and bladder dysfunction (Noseworthy et al., 2000). Ocular manifestations often present early in the disease course and are typified by loss of visual acuity and painful ocular movements in this subset of patients (Soderstrom et al., 1998). Alterations in PTMs of proteins may represent some of the critical changes in the early pathogenesis of MS, as improper immune activation plays an important role in the disease process (Arnon and Aharoni, 2007; Ziemssen, 2005). Deimination was demonstrated to be one of the important PTMs in the development of MS given that brain tissue samples from MS patients showed hyper-deiminated
regions and elevated levels of PAD2 when compared to normal controls. MBP derived from MS patients is heavily deiminated (Moscarello et al., 1994) and shows enhanced susceptibility to both autocatalytic (D'Souza et al., 2005) and cathepsin D-mediated proteolysis (Pritzker et al., 2000). These proteolytic products have the ability to sensitize T-cells (Belogurov et al., 2008; D'Souza et al., 2005) and may also activate astrocytes that have been implicated in both innate and acquired immune responses in the CNS (Nair et al., 2008).

1.3 Transgenic mouse model (ND4) of multiple sclerosis. Animal models of MS facilitate modification of environmental factors and help elucidate the role that these factors play in disease progression. The basic mechanisms underlying oligodendrocyte development and myelination are closely conserved from mouse to human. As a result of the availability, lifespan, and fecundity of rodents as well as the resemblance of experimental autoimmune encephalitis (EAE) to human MS, mice serve as an especially valuable model system for MS studies. The transgenic mouse model of MS (ND4) has been previously utilized to evaluate retinal deimination levels (Mastronardi et al., 1993). ND4 mice contain 70 copies of the transgene for DM20, a proteolipid that was reported to play an important role in the onset of myelinogenesis (Ikenaka et al., 1992). DM20 is a major proteolipid in juvenile mice, but high DM20 protein levels in adult mice results in abnormal myelin assembly with a high propensity for disruption. Thus, the persistence of immature myelin into adulthood induces demyelination in ND4 mice (Enriquez-Algeciras et al., 2011). In animal models, changes in spasticity and gait abnormalities are utilized as clinical symptoms of demyelinating disease
ND4 mice appear normal up to 3 months of age but develop a wobbling gait with tremors and seizures by 8-10 months of age.

1.4 The paradox of deimination in diseases and aging. Normal retinas show deimination in most of the retinal layers but also demonstrate layer-specific and age-specific variations in the pattern of deimination. For example, hyper-deimination is observed in CNS tissue from young children but not adults. Likewise, a mouse model parallel aging study using the F1 hybrid between Fischer 344 and Brown Norway rats (F344BN) demonstrated that aged rats (~24 months) showed decreased deimination in comparison to young rats (~3 months). Immunohistochemistry performed on retinal cryosections showed that the ganglion cell layer undergoes significant, age-related loss of deimination (Bhattacharya et al., 2008). This decrease was also significant in the inner plexiform and inner nuclear layers, and the observed decrease in deimination for F344BN retinas was commensurate with an observed decrease in PAD2 mRNA levels and expression products (Bhattacharya et al., 2008). Conversely, aberrant ocular deimination, especially in the form of global hyper-deimination, has been associated with neurodegenerative diseases (Bhattacharya et al., 2006c; Nicholas et al., 2005; Proost et al., 2008). Elevated levels of PAD2 and protein deimination have been found in adult-onset diseases such as rheumatoid arthritis (Scofield, 2004) and in several human neurological diseases that include MS (Moscarello et al., 2002), autoimmune encephalomyelitis (Nicholas et al., 2005), Alzheimer’s (Maruyama et al., 2005; Louw et al., 2007), amyotrophic lateral
sclerosis (Chou et al., 1996) and glaucoma (Bhattacharya et al., 2006a, b). Aging studies with F344BN rats (Bhattacharya et al., 2008) therefore suggest that the elevated levels of PAD2 and deiminated proteins seen in late-onset, progressive ocular diseases such as glaucoma, MS and EAE (Bhattacharya et al., 2006a; Bhattacharya et al., 2006c) are likely due to the pathological process of disease rather than age-associated changes.

In an additional complication, we observed hypo-deimination in the RGC layer, with hyper-deimination of the inner nuclear layer and the inner aspect of the outer nuclear layer as observed by the absence of fluorescent spots in the ND4 mice compared to the controls (Figure 2.1). The observation of hypo-deimination in the RGC layer is in contrast to known hyper-deimination in the brains of ND4 mice and deceased human MS patients (Mastronardi et al., 2007; Moscarello et al., 1994). A similar loss of deimination has been observed in the RGC layer of MS tissue donors compared to controls, a phenomenon not due to a general loss of RGC neurons (see results).

Modulation of deimination levels has yet to be ascribed to a specific physiological condition. It is important, therefore, to establish the events associated with decreased deimination observed in the RGC neurons of MS patients. It is also possible that decreased deimination levels observed in neurons from aged animals may result in some functional impairment. We propose that this paradox may be caused by contributions from different cell types.
1.5 **Dendritic Protein Synthesis.** The transport and localization of specific mRNAs to dendritic spines has recently received attention following the discovery of activity-dependent localization of mRNAs in dendrites of cultured neurons. This has been suggested as an important mechanism in the regulation of local synapse development and synaptic plasticity (Tiruchinapalli et al., 2003), and RNA binding proteins may be important mediators of local dendritic protein synthesis given their potential role in transporting mRNAs to dendrites of activated synapses. One well studied example is the localization of zipcode binding protein 1 and β-actin mRNA in dendrites of hippocampal neurons. Zipcode binding protein has been found to bind β-actin mRNA and is regulated during synapses formation (Eom et al., 2003). These studies suggest the existence of a local control system for dendritic protein synthesis, and our work explored deimination as an important regulator of these synthetic pathways.

1.6 **Mitochondria and MS, Mitochondrial mRNA transport.** Mitochondria (mt) are major energy providers for the cells and are involved in a variety of basic cellular processes in higher organisms. Crosstalk between the nucleus and mt plays an important role for mt genome integrity and mt biogenesis. Translation of nuclear-encoded mt mRNA occurs in the cytoplasm, with subsequent assisted transport of the nascent polypeptides via the mt translocation complexes (Franke et al., 2001; George et al., 1998). In yeast, some mt proteins have demonstrated co-translational import via surface-bound ribosomes (Fujiki and Verner, 1991; Fujiki and Verner, 1993), indicating the presence of another protein transport mechanism based on the existing mRNA on the mt surface [reviewed in (Ding et
Several nuclear-encoded mRNAs such as ATP2 and ATM1 have been shown to be transported onto the mt surface (Beddoe and Lithgow, 2002; Margeot et al., 2002). The transport of mRNA to the mt surface, local protein synthesis, and subsequent mt import are essential processes for mt structural integrity and function.

Neurons are extremely vulnerable to mt defects, and the importance of mt is underscored by the fact that a number of mtDNA mutations present as neurological diseases/disorders, including those with early ocular manifestations. In many neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis, secondary forms of oxidative phosphorylation (OXPHOs) deficiency have been detected in addition to mt protein abnormalities.

In MS and optic neuritis, mitochondria have been implicated in the pathogenic process (Kalman, 2006). Mutations in mt NADH dehydrogenase 4 and 6 have been identified in patients with MS and/or optic neuritis, with proposed pathologic significance for blindness. Most mtDNA mutations locate to subunits of the respiratory chain Complex I. Mice with disrupted NADH dehydrogenase alpha 1 (NDUFA1, a subunit of Complex I) demonstrated severe optic nerve degeneration, while several mutations in nuclear DNA-encoded subunits of respiratory chain enzymes have been found associated with Leigh-like syndrome (Kalman, 2006).

Each mt has multiple copies of circularized DNA that encode 2 ribosomal RNAs, 22 transfer RNAs, and 13 protein genes. However, the synthesis of most
mt proteins is also dependent on genes encoded by nuclear DNA. Mitochondrial defects can initiate from multiple sites of mutation occurring in both mt and nuclear DNA, which can lead to defects such as decreased ATP synthesis, oxidative stress, increased ROS production, and the initiation of apoptosis.

While most studies have focused on genetic mutations, inefficiencies in the transport of nuclear-coded mt mRNA have yet to be investigated as pathogenic in neurological disorders. What is the role of deimination in mt surface protein translation? This study may provide a basis to formulate such experiments in the future.

1.7 RNA Export and Binding Proteins (REF). Cytosolic translation necessitates a number of accessory proteins, including those responsible for nuclear mRNA export. Preliminary studies using retinal extract or isolated RGCs revealed a protein of approximately 22-30 kDa that was enriched during immunoprecipitation with anti-citrulline. Based on the source tissue, this protein was identified by mass spectrometry as human REF or one of its murine analogs. Members of the REF family consist of a central RNP-type RNA binding domain flanked by Ala/Arg/Gly-rich regions of variable length (Nojima et al., 2007). In humans, REF is also referred to as THO complex 4, Aly and BEF (bZIP enhancing factors), and was found to be ubiquitously expressed in retinal and neural tissues. This protein is known to function as a chaperone and promotes transcriptional activation via facilitated dimerization of transcription factors containing basic leucine zipper (bZIP) motifs. It also plays a role in mRNA processing and export. As its name implies, REF is an integral part of the
THO/TREX complex that is recruited to actively-transcribed genes and travels with the RNA polymerase during elongation. It has been demonstrated that REF remains associated with spliced mRNA and plays an important role in mRNA export, especially as it relates to retrotransposition suppression (Nojima et al., 2007). It has also been proposed that REF may be a scaffold that mediates interactions between proteins and/or RNA. In contrast to studies in the nucleus, the role of REF in the cytoplasm is not clear. In mice, there are two REF homologs referred to as REFBP1 (long form) and REFBP2 (short form). Our initial studies suggest that deimination of REF occurs in human retinas, and both REFBP1 and REFBP2 show evidence of deimination in mice retina. REF was found to be one of the major targets of deimination in neuronal cells in this study (see results) and was further applied as a mediator to study the different roles of deimination. In this dissertation, REF in mouse model studies is referred to REFBP2 unless specified.
CHAPTER II: Local retinal hypo-deimination and decreased dendritic protein synthesis

2.1 Overview

In contrast to global hyper-deimination in the brain and eyes, we found local hypo-deimination in the retinal ganglion cell (RGC) layer from MS patients and in a transgenic mouse model (ND4) of MS. REF, an RNA-binding/export protein, was specifically found to undergo loss of deimination in MS, resulting in functional changes in RNA binding efficiency. Only the deiminated form of REF interacts with the eIF4F complex and enhances local translation of SNAP-25 mRNA transcripts in neuronal dendrites. Restoration of deimination in the optic nerve results in dramatically improved visual function and continued neurite extension in isolated neurons. Down-regulation of either deimination or REF results in decreased neurite outgrowth. Together, these findings support a key role for deimination in dendritic protein synthesis and reveal a new pathogenic mechanism occurring in the early stages MS as well as other neurodegenerative diseases.

2.2 Results

2.2.1 Detection of loss of deimination in ND4 mouse retinal ganglion cell (RGC) layer. We detected general hyper-deiminated regions in MS as compared to normal brains (Figure 2.1A, A’) consistent with increased deimination of MBP. In contrast, we found hypo-deiminated regions in the RGC layer of MS retinas relative to the hyper-deiminated regions found primarily in the inner and outer nuclear layers and in the outer plexiform layer (Figure 2.1B, B’).
Deimination was evaluated in a transgenic mouse model of MS (ND4) that over-expressed the DM20 variant of myelin proteolipid protein and presented many features of the disease (Johnson et al., 1995; Mastronardi et al., 1993). Similar to the pattern in human MS, we observed hypo-deimination of the RGC layer with hyper-deimination of the inner nuclear layer, outer plexiform layer, and the inner aspect of the outer nuclear layer (Figure 2.1C, D). However, significant decreases in the numbers of Thy1 or MAP2 neurons were not detected in either human MS retinas (Figure 2.1E and Table 2.1) or in ND4 mouse retinas (Figure 2.1F) as compared to controls. The observed hypo-deimination was detected in a significant number of cells as early as 3 months - notably, prior to the appearance of clinical symptoms (Figure 2.1G). In normal retina, cells harboring deiminated proteins in the RGC layer have a characteristic small-molecule signature (Figure 2.2 A-F) that is typically associated with ganglion cells (Marc and Jones, 2002), although a weak deimination signal can occasionally be found in displaced amacrine cells of the ganglion cell layer (Figure 2.2 C-F, cells “I”). Cytochemical analysis of first-passage, mixed-population retinal cell isolates showed no or exceedingly low levels of deimination in cells positive for the amacrine marker HPC1 but negative for RGC markers such as Thy1, γ-synuclein, or NeuN (not shown), supporting the preferential localization of deiminated proteins to RGCs.

2.2.2 Identification of RNA Export Factor (REF) as a target of deimination.

We next determined the identity of the major deiminated proteins in RGCs. Immunoprecipitation and subsequent mass spectrometry/Western analysis of
deiminated proteins from total retinal lysates extracted from normal mouse, human, and pig eyes consistently identified the RNA-binding and export protein REF (Golovanov et al., 2006; Rodrigues et al., 2001) or its mouse homolog REFBP2 (Figure 2.3A). In comparison to wild type mice, Western analysis of retinal protein from ND4 mice showed decreased deimination of REF BP2 (Figure 2.3B), complementary and consistent with immunohistochemical findings (Figure 2.1) as well as several other techniques (described below). Next, we cloned histidine-tagged REFBP2 (Figure 2.3C) to demonstrate binding affinity for RNA in both its deiminated and non-deiminated forms (Figure 2.3D). However, deiminated REFBP2 promoted the selective enrichment of certain mRNA species (Figure 2.3D). Subsequent microarray analysis of these species identified a number of SNARE complex components (Table 2.2; GEO accession number GSE11843) as well as nuclear-encoded mt components, (see part II and Table 3.1).

2.2.3 Increased dendritic protein synthesis with deiminated REF BP2. In vitro binding assays using deiminated REF BP2 resulted in enrichment of SNAP-25, VAMP2, and complexin1 mRNA. Interestingly, SNAP-25 has been shown to play a critical role in neurite elongation (Osen-Sand et al., 1993). Binding and enrichment of SNAP-25 mRNA by deiminated REF BP2 was further verified using UV cross-linking of protein and mRNA as well as northern analysis. Furthermore, a 6xHis-tagged TUNP (transformation up-regulated nuclear protein - an RNA binding protein) control under identical conditions showed no affinity for SNAP-25 mRNA (Figure 2.3E). We then determined whether binding to deiminated
REFBP2 modulated SNAP-25 translation. A preparation of a translationally-competent protein extract (free of polyadenylated RNA and SNAP-25 protein) from approximately 225,000 laser-captured RGC dendrites showed enhanced translation of introduced SNAP-25 mRNA (with subsequent SNAP-25 protein accumulation) in the presence of in vitro-deiminated, recombinant REFBP2 as compared with non-deiminated REFBP2 (Figure 2.3G). Biochemical studies revealed a greater SNAP-25 mRNA dissociation constant for non-deiminated REFBP2 compared with deiminated REFBP2 (data not shown), suggesting that deimination alters the RNA binding properties of REFBP2, strengthens SNAP-25 mRNA binding, and enhances dendritic protein translation of select mRNAs.

2.2.4 Decreased neurite outgrowth with deimination inhibition. We then investigated whether the principal deiminating enzymes PAD2 and PAD4 affect neurite elongation by modulating local dendritic protein synthesis. We excluded an effect of other PAD enzymes: PAD1 and PAD3 were not detected in RGCs at either the mRNA or protein level (data not shown), and PAD6 was found to lack enzymatic activity (Raijmakers et al., 2007). PAD2/PAD4 siRNA knockdown (individually and in combination) significantly decreased neurite length compared with control siRNA in rat RGC neurons (Figure 2.3H). Similarly, PAD2/PAD4 siRNA decreased neurite length in other types of rat neurons (see part II). Consistent with a local effect on neurite outgrowth, immunofluorescence analysis detected deiminated proteins in dendrites undergoing outgrowth (Figure 2.3I, J), and treatment of RGCs with shRNA targeting the REF coding region resulted in decreased neurite outgrowth compared to controls (Figure 2.3F). Thus, a
decrease in both deimination (Figure 2.3H) and REF (Figure 2.3F) reduced neurite outgrowth, supporting both the regulation of neurite elongation by REF and the deimination-based modulation of dendritic protein synthesis.

2.2.5 Detection of deimination residues in REFBP protein. Several different techniques further supported the loss of deimination in ND4 mice retinas (Figure 2.1C-G) and in particular, the loss of REFBP2 deimination (Figure 2.3B). Amino acid analysis of hydrolyzed REFBP2 purified from neurons (cytosolic fraction; wild type mouse) demonstrated the conversion of up to 7 arginine residues into citrulline (Table 2.3). Mass analysis of intact REFBP2 isolated from wild type mice by linear-mode mass spectrometry (Figure 2.4A) was consistent with the amino acid analysis. Using high-resolution mass spectrometry, we further identified 7 arginine deimination sites in REFBP2 from wild type mice (Figure 2.4B-C) that demonstrate loss of deimination in ND4 mice. Several different optimization protocols and proteases were utilized to identify deimination sites, and pepsin was found to provide the best coverage. We consistently and frequently found a deiminated peptide fragment corresponding to R20-A34 of REFBP2 (RRVNRGGPRRNRPA). The arginine residues shown in bold (* in Figure 2.4B) were identified as deiminated, while the bold italicized (**) in Figure 2.4B) arginine was found either deiminated or methylated (not shown) in different REFBP2 molecules. The non-deiminated and deiminated peptides from REFBP2 co-eluted, co-migrated, and were often found in equal ratios. A region of the spectra corresponding to the fragment RRNRPA from this peptide was provided (Figure 2.4B). Corresponding Y-ions of the R30 and R32 arginines were
detected (+1 m/z) for Y-5 and Y-7 (Figure 2.4B). The detected Y and b ion m/z ratio (Figure 2.4C) and the sequence have been provided (Figure 2.4D). REFBP2 was also modeled with PyMol using the NMR structure obtained from the RCSB Protein Data Bank archive (accession number 2F3J) corresponding to SwissProt accession number Q9JJW6 (Figure 2.5). Based upon our model, an N-terminal alpha-helical region spans residues 8-19. This helix has an overall negative charge except for the terminal region due to the influence of the positively-changed R17. This arginine is subject to deimination, and the loss of charge appears to change the secondary structure of this region. The relaxed conformation that results from this change in folding state may better enable REFBP2 to accommodate substrate RNA. However, the most prominent change occurs at R24. In solution, R24 faces R37, a highly–repulsive and destabilizing interaction (Figure 2.5A, B), and deimination of R24 is expected to render this interaction energetically favorable. Furthermore, deimination of R21, with its neighboring proline residues, should result in a more rigid structure and increase the RNA binding affinity. V8 protease digestion also suggests that the linker region confers some flexibility to the local structure. Thus, deimination of REFBP2 arginines is expected to have consequences for both structure (Figure 2.5) and function.

2.2.6 Increased neuron function after restoration of deimination in ND4 mice. Like MS patients (Kolappan et al., 2009; Xu et al., 2008), ND4 mice suffer from a loss of visual function (Enriquez-Algeciras et al., 2011) (Figure 2.6) that precedes the more obvious clinical symptoms of spastic tremor and gait
abnormalities that occur around three months of age. We found that ND4 mice and their non-transgenic counterparts exhibited similar Pattern electroretinograms (PERGs) and Flash electroretinograms (FERG) until about 3 months of age, at which point PERG amplitude began to decrease in ND4 mice while FERG remained the same (Enriquez-Algeciras et al., 2011). PAD2 expression under the control of the Thy1 (Figure 2.7) or Brn3b promoter (enabling relatively specific expression of PAD2 in RGCs) resulted in the recovery of PERG amplitude (Figure 2.7 A, B). ND4 mice with exogenous PAD2 expression in the optic nerve displayed a greatly reduced (>30%) decline in PERG amplitude as compared to controls expressing GFP (Figure 2.7C). Concurrently, PAD2 expression led to increased elongation of neurites in isolated neurons relative to non-transfected controls (Figure 2.7D, E).

2.2.7 The role of deimination in dendritic protein synthesis. To determine whether deiminated REFBP2 interacts directly with the cellular translation machinery, we immunoprecipitated recombinant REFBP2 (with or without deimination) containing SNAP-25 transcripts from the fractionated cytosolic extracts of isolated RGCs. From the results, it was discovered that deiminated REFBP2 interacts with translation initiation complex proteins (Figure 2.8A). A complex freed from eIF4B was precipitated using anti-REF and divided into two fractions. Each fraction was spiked with either purified CRALBP (cellular retinaldehyde binding protein; control) or eIF4B, and both fractions were combined with equal amounts of SNAP-25 mRNA. A second IP with anti-REF enabled PCR amplification of SNAP-25 from the CRALBP-containing fraction but
not the eIF4B-containing fraction (Figure 2.8B). These results show that deiminated REFBP2 directs the delivery of SNAP-25 and VAMP2 mRNA to the ribosomal translational machinery. Here, we propose a mechanism for the sequestration and delivery of mRNA (such as SNAP-25) by deiminated REF for the synthesis of dendritic proteins (Figure 2.8C) This is consistent with previous demonstrations of elevated local dendritic protein synthesis following the delivery of SNARE complex mRNA (SNAP-25/VAMP2) via deiminated REFBP2 (Sutton and Schuman, 2006). Conversely, loss of mRNA transport explains local translation failure of SNARE complex proteins as well as other proteins critical for neurite elongation/outgrowth (Shelly et al., 2007). The results further suggest that the loss of deiminated REFBP2 in MS facilitates decreased protein synthesis, increased disassembly, and perhaps even retraction among some RGC neurites at an early stage in the disease process. The loss of deiminated REFBP2 in pathogenic states would most certainly result in decreased SNAP-25 transcripts in neuronal dendrites. Indeed, neurites captured from freshly-isolated RGCs derived from the eyes of ND4 mice show lower ratios of SNAP-25 mRNA when normalized against β-tubulin mRNA from control C57BL6/J mice, suggesting that ND4-derived RGC neurites contain lower levels of SNAP-25 mRNA (Figure 2.9). The loss of deimination likely favors a shift in the assembly/disassembly equilibrium of dendrites toward disassembly, as mediated by the regulated synthesis of proteins involved in constitutive exocytosis/endocytosis.
2.3 Discussion

The early loss of deimination detected in the ND4 mouse retinas may be the result of chronic stress experienced by neurons in a pathologic, and perhaps inflammatory, state. This loss of deimination could decrease local dendritic protein synthesis and lead to a functional impairment of neuronal connections as a direct result of neurite disassembly. Relative loss of deiminated RGCs has also been detected in the myelin oligodendrocyte glycoprotein-immunized mouse model of MS (Sun et al., 2001), in glaucomatous human eyes (Table 2.1), and in the DBA/2J mouse model of pigmentary glaucoma (John et al., 1998, not shown). The elevated intraocular pressure seen in glaucoma often acts as a chronic stressor for RGCs. To determine whether chronic stress results in a loss of deimination and decreased neurite outgrowth, isolated rat RGCs were subjected to elevated pressure using established methods (Bhattacharya et al., 2006b). Chronically-elevated pressure was shown to decrease both deimination and neurite outgrowth (Figure 2.9). Likewise, RGCs subjected to chronic centrifugal force have shown a similar decrease in neurite outgrowth (Kashiwagi et al., 2004). It is important to note that RGCs and astrocytes show opposite PAD2 expression patterns in response to pressure (and other stressors); whereas PAD2 is elevated in response to pressure in astrocytes, it is down-regulated in RGCs (Bhattacharya, 2009). Hyper- and hypo-deimination occur simultaneously in neuronal tissues. However, retinal hypo-deimination is more discernible in the RGC layer due to the lack of astrocytes. Elevated PAD2 and
concurrently-increased deimination in large number of astrocytes render detection of neural hypo-deimination extremely difficult in the brain.

In summary, we demonstrate that a decrease in REF and deimination results in reduced neurite outgrowth; conversely, restoration of deimination prevents the correlated loss of vision and is commensurate with increased neurite outgrowth in isolated neurons.

Decreased deimination is associated with normal aging (Bhattacharya et al., 2008), whereas increased deimination is seen in neurodegenerative diseases such as MS (Moscarello et al., 1994) and glaucoma (Bhattacharya et al., 2006b). However, a loss of deimination is observed in RGCs in MS (Figure 2.1E-G) and glaucoma (not shown) - although it occurs earlier and at a faster rate than in normal aging. In addition to regulating normal dendritic protein synthesis, deimination appears to be involved in cold-inducible dendritic translation of mRNA. The cold-inducible mRNA-binding protein RBM3 has been reported to localize to neuronal dendrites, undergo an unknown posttranslational modification, and promote translation (Smart et al., 2007). We have identified deimination of RBM3 arginine residues in RGCs (Enriquez-Algeciras et al., unpublished observations). Similar to the arginine-rich linker region of REFBP2 that is necessary for RNA binding (Rodrigues et al., 2001), RBM3 has been shown to possess equivalent RNA-binding arginine residues (Smart et al., 2007) that are possible targets for deimination. Regulation of local dendritic protein synthesis by deimination may be a feature of other neurodegenerative diseases that present ocular manifestations as well.
Figure 1.1 Reaction of deimination.
Figure 2.1 Relative hypo-deimination in the RGC layer in MS. (A) Deimination detection from (A, A') normal and MS human brains and retina (B, B'). Thin and thick arrows indicate normal deimination and hyper-deimination in ganglion cell and other cell layers, respectively. (C) Retinas from (5-month-old) control mice and (D) age-matched ND4 mice with identical backgrounds were subjected to monoxime treatment. Deimination was detected using an anti-citrulline-monoxime adduct antibody. Yellow thin arrows (C) indicate a subset of RGCs that is deiminated (green). One spot indicated by the box and shown in the inset is absent in ND4 mice (D). Panels C' and D' depict similar regions without monoxime treatment that were subjected to anti-citrulline-monoxime adduct immunofluorescence. Blue arrows (D) indicate hyper-deiminated regions. There is no loss of RGCs in ND4 mice compared to controls (C', D'). Arrowheads show nuclei of RGCs -DAPI (blue). GCL=ganglion cell layer; IPL=inner plexiform layer; INL=inner nuclear layer; OPL=outer plexiform layer; ONL=outer nuclear layer; PR=photoreceptor layer. Bar= 50µm. (E) Determination of citrullinated RGC numbers in 0.1 sq. mm in human retina from 5 control and 5 MS eyes. (F) Determination of Thy1 and MAP2 marker intensity in equal areas in RGC layer in control and ND4 mouse at 5 months of age, normalized for DAPI staining within the equivalent regions of retina under measurement as described in methods. (G) Determination of ratio of anti-citrulline positive cells per Thy1 positive RGC cells of ND4 and control mice from equivalent regions of retina. Mean ± SD from at least three independent measurements has been shown. All data subjected to two-tailed paired or unpaired t-test with respect to controls (*), **P≤0.05.
Figure 2.2 Validation of deiminated retinal ganglion cells using small molecule. (A) Multispectral visualization of citrulline (c, red channel), GABA (g, green channel) and glutamate (E, blue channel): xgE :: rgb mapping. RPE retinal pigmented epithelium, OSL outer segment layer, ONL outer nuclear layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer. The box around cells in the GCL is 90 um wide. (B) The the citrulline greyscale channel. (C) Enlargement of the boxed region in A using xgE::rgb mapping. Circled cells 1-9 are several different kinds of retinal ganglion cells, all ganglion cells have strong deimination signals (D) varied GABA signals based on heterologous coupling (E) cells 4 and 8, and strong glutamate signals (F) Cells labeled "i" are displaced amacrine cells and have measurably weaker citrullination (about 2-fold less).
Figure 2.3 Identification of REFBP2 and RNA binding. (A) Deimination detection of immunoprecipitation (IP) products using anti-citrulline antibody by Western analysis, lane 1 (S), the RGC lysates, lane 2 (+), anti-citrulline-IP. The arrow indicates the region of the gel that was subjected to mass spectrometry. (B) Lysates from ND4 and control RGCs were gel separated, protein bands corresponding to REF (~23KDa) region were excised and reloaded onto a fresh SDS-PAGE (upper panel), and detected using anti-REFBP2 and anti-citrulline antibodies. (C) Cloning of His-tagged REFBP2 protein into E.coli [lane1 (-), without induction; lane 2 (+), with induction, lane3 (P), purified recombinant REFBP2]. (D) RNA binding assay. (S): total RNA from mouse brain; (-d) and (+d): bound RNA species eluted from non-deiminated and deiminated REFBP2. Arrowhead indicates enriched RNA species. (E) Purified His-tagged TUNP or REFBP2 (top panel) was UV cross-linked with the total RNA pool and subjected to PCR amplification of SNAP25 mRNA (bottom panel). (F) The effect of siRNA (against PAD2 and PAD4) treatment on neurite outgrowth in RGC neurons. Average neurite length (40 neurons x 10 neurites) is shown (µm). (G) The laser-captured neuronal dendrites were used for preparation of a translation-competent extract after depletion of total mRNA and added to SNAP25 mRNA and non-deiminated (-d) or deiminated (+d) REFBP2, as indicated. (H) The effect of shRNA (against REF mRNA) treatment on neurite outgrowth in cultured RGCs. Average neurite length (same as above) is shown (µm). (I) Localization of deiminated proteins in cultured mouse primary RGCs and (J) REFBP2 (red) and deimination (green) in elongated neurites, DAPI (blue) indicates nuclei. Arrows indicate co-localization of deimination and REFBP2. Mean ± SD from at least three independent measurements has been shown. All data were subjected to two-tailed paired t-test with respect to controls (*), **P≤0.005.
Figure 2.4 Identification of REFBP2 deimination sites. (A) Mono Q and Mono S FPLC column purified hippocampal REF (Q9JJW6) showed a mass of 23730.29 corresponding to unmodified REFBP2 and a large peak for 23737.32 corresponding to deiminated REF for 7 arginine residues. The amino acid analyses of bands with pepsin digestion confirmed them as REFBP2. The deiminated band citrulline yield was consistent with 7 residues modified. Additional arginine modifications (methylation) cannot be ruled out. (B) REFBP2 deiminated arginines are shown in bold (*). The bold italicized (**) arginine indicates deiminated or methylated. The RNA binding pocket is underlined. Annotated spectrum of RRVNRGGGPRRPNARPA from the pepsin digested REFBP2 and the magnified view of Y7 ion is shown in the inset. M +1Da ion. (C) The Y5 and Y7 ions showing M+1Da ions. (D) The experimental b and y ions of the six amino acid sequence containing the deimination sites. The identified M+1Da ion corresponding to Y-ions have been shown as underlined italicized numbers.
Figure 2.5 REF modeling using PyMol Program. (A) The modified residues are identified using ball and stick. (B) The charge distribution in native (unmodified) REF. Arginine residues 24 and 37 are face to face (arrow), which is a highly repulsive destabilizing interaction. NMR structure accession number 2F3J corresponding to SwissProt accession number Q9JJW6.
Figure 2.6 Determination of flash (FERG) and pattern (PERG) electroretinogram in ND4 mice. The dashed line and hollow triangle and solid line and triangle represent FERG and PERG amplitudes respectively as indicated. Age of mice has been indicated (n= 10 mice per group). Note that where as FERG amplitude remains unaltered the PERG amplitude undergoes a significant decrease indicating an impairment of visual function predominantly due to impaired RGC function.
Figure 2.7 Restoration of deimination and increased visual function in ND4 mice. (A) Representative pattern electroretinogram (PERG) signal from an ND4 mouse, pre-injection at 3.5 months; (B) the same mouse eyes at 15 days post-injection; the left (PAD2-expressing) eye is shown by solid lines and the right eye expressing control GFP is shown by dashed lines. (C) PERG amplitude normalized to baseline for PAD2-expressing and control GFP-expressing (sham) animals for 7 injected mice at 15 days (hollow bars) and at 30 days (dotted bars). Each bar represents mean± standard deviation of all recordings and was found to be significantly different from 0.0 at each time point by the one-sample t-test (*P ≤ 0.02). (D) Isolated RGCs from ND4 mice and (E) RGCs transfected with PAD2 lentiviral construct and incubated for identical periods. The cells were probed with anti-Thy1, anti-REF and anti-citrulline as indicated. Bar=10µm.
Figure 2.8 Effect of deimination on dendritic protein synthesis. (A) Western analyses of products from immunoprecipitation with anti-REFBP2 demonstrate that deiminated REFBP2 (+d) but not control non-deiminated REFBP2 (-d) interacts with PABP-1 and elf4E (elf4F complex). The deiminated REFBP2 also interacts with elf4B, which subsequently results in release of polyadenylated mRNA to the ribosomal complex. (B) The products from anti-REF IP were subjected to subsequent IP with elf4B and removal of total mRNA by an oligodT column. An equal amount of SNAP 25 mRNA was put in two equal fractions and incubated at room temperature for 10 minutes. Subsequently, 0.1 µg of purified elf4B or CRALBP was added and then the mixture was subjected to IP with anti-REF. The PCR reaction amplified SNAP 25 from the immunoprecipitation that included CRALBP but not from the immunoprecipitation with purified elf4B. Bottom panel shows results obtained after probing the fractions with anti-REF. (C) Model for interaction of deiminated REFBP2 with ribosomal complex. The deiminated REFBP2 [white ellipsoid with bound polyadenylated RNA; (d) indicates deimination] exports polyadenylated cargo from the nucleus and becomes additionally deiminated (indicated by dd). As shown by Western analyses in (A), the deiminated REFBP2 interacts with PABP-1 and elf4E, two members of the elf4F complex. The elf4F complex is known to interact with the 43S subunit, resulting in formation of the 48S ribosomal subunit. elf4B interacts with this complex (shown by Western analysis), leading to the release of bound, polyadenylated RNA to the
Figure 2.9 Reduced deminination and protein translation as a consequence of insults. (A) Determination of levels of SNAP25 and β-tubulin mRNA in laser captured retinal ganglion cell neurites. The mRNA was extracted from 1000 laser captured neurite each from approximately ten isolated cells (selected arbitrarily) and subjected to probe for SNAP25 and β-tubulin using non-radioactive labeling. The plot shows quantitative measurement from conjugated chemiluminescence (as described in methods) attached to the non-radiolabeled probe for SNAP25 and β-tubulin. The last two bars shows the ratio of chemiluminescence from SNAP25/β-tubulin. The results are from three independent experiments, standard deviation has been presented. (B) Retinal ganglion cells at two days isolated from rats were subjected to pressure in a chamber (as described in methods). The cells were subjected to pressure for about 16 hours or 8 cycles of pressure for 2 hours pressure and 1 hour atmospheric pressure. The neurite lengths from an average of 10 cells were measured by two independent observers. Mean±SD from three independent measurements have been presented. All data were subjected to two-tailed paired t-test against controls (*), **p≤0.05.
Table 2.1 Donor information

**Multiple Sclerosis donors**

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* Our internal sample identification number, W indicates Caucasian. **C/D = Cup to disc ratio. POAG= primary open angle glaucoma. Clinical diagnosis and Glaucoma scaling is based on a static perimetry threshold test (24-2), glaucomatous hemifield test and
### Table 2.2 Select REFP2 bound RNA (SNARE complex members) identified by Affymetrix microarray analyses

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*Total input isolated mouse brain RNA; **REFP2 bound eluted RNA from two identical binding experiments indicated by (1) and (2).
Table 2.3 Amino acid analyses of Recombinant and purified REF proteins

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The recombinant REF (REFBP2) had a tag: MGHHHHHHHHHHSSGHIDDDDKH. **

Theoretical estimates. The amino acid analyses was performed on a Hitachi L-8900 amino acid analyzer. The analysis was performed using 25µg of protein in a 50µl volume. Approximate molar percent (Approx. %) was determined and number of arginine residues or citrulline residues were calculated from these determination.
3.1 Overview

Deimination refers to the conversion of protein-bound arginine into citrulline. Mitochondrial RNA binding export factor (REF) undergoes loss of deimination that results in impaired ATP5b mRNA transport in ND4 mice (model of multiple sclerosis). We present evidence that (1) non-deiminated REF has reduced ATP5b mRNA binding affinity compared to deiminated REF, (2) ND4 mice have impaired ATP5b mRNA transport and, (3) PC12 cells exhibit reduced mitochondrial ATP synthase activity upon inhibition of deimination. Impaired deimination of REF and defects in mitochondrial mRNA transport are critical factors leading to mitochondrial dysfunction in ND4 mice.

3.2 Results

3.2.1 Evidence of REF association in ATP5b mRNA transport. Cytosolic and mitochondrial protein extracts were prepared from ND4 and wild type control mice retinas following established protocols. We performed immunoprecipitation (IP) to determine the presence of REF in mitochondrial and cytosolic fractions, and it was found to be present in both fractions (Figure 3.1A) and was associated with different mRNAs. Negative control IP with anti-aldehyde dehydrogenase (ALDH1) showed lack of REF pull-down (Figure 3.1A-c’). We identified a number of nuclear-encoded mitochondrial mRNAs, especially a series of ATP synthase complex mRNAs, which showed significantly increased binding with deiminated REF compared to control REF (Table 3.1 online, GEO
accession number GSE11843). Mitochondrial mRNAs have been shown to be transported to the mitochondrial surface and locally translated (Corral-Debrinski, 2007; Corral-Debrinski et al., 2000; Kellems et al., 1975), including the yeast mRNAs ATP2 and ATM1 (Corral-Debrinski et al., 2000). We sought to determine whether both ATP5b (mouse homolog of ATP2) and ATM1 bind to REF intracellularly, and the existence of both ATP5b and ATM1 mRNA at the mt surface was confirmed using RT-PCR (Figure 3.1B-a’). IP of REF-associated protein/mRNA complexes was carried out with two different mt fractions derived from synaptic and non-synaptic environments as well as the cytosolic fraction. ATP5b and ATM1 mRNAs from the eluents were detected by PCR (as described above) (Figure 3.1B-b’), corresponding to the demonstration of ATP5b shown in both cytosolic and mitochondrial fractions (Figure 3.1B-b’). In contrast, actin mRNA (control) and ATM1 were detected only in the cytosolic but not in mitochondrial fractions (Figure 3.1B-b’). Detection of actin and ATM1 in cytosolic fractions supports the mRNA export function of REF in the cytosol, where REF is expected to associate with most mRNAs. However, our PCR analyses revealed a stronger association between REF and ATP5b compared to that of ATM1 mRNA at the mt surface, suggesting that ATM1 and ATP5b may be transported by two different pathways. Regardless, these results suggest that REF is involved in the transport of ATP5b.

3.2.2 Localization of REF at mitochondrial surface. Localization of REF to the mt surface was detected by immunohistochemistry, confocal microscopy (Figure 3.2) and electron microscopy (EM) (Figure 3.3). Mt surface markers Porin/VDAC
and Tomm20 (Figure 3.2) were used to observe REF co-localization at the mt surface in primary neurons isolated from rat hippocampi, and REF was detected with multiple antibodies [Figure 3.2 A-C, rabbit polyclonal anti-REF (Aviva systems biology); Figure 3.2 A’-C’, mouse monoclonal anti-REF (Abcam)]. Along with mt surface localization, REF was found to be present at other locations in elongating neurites (Figure 3.2). Consistent with the confocal microscopy data, EM images also showed localization of REF at the mt surface (Figures 3.2; 3.3 C, D). Primary control EM sections showed a continuous presence of gold particles (Figure 3.3 A, B).

3.2.3 Reduced level of deimination of REF in ND4 mice. Western analyses were consistent with a loss of REF deimination in the ND4 mouse model compared to the normal controls (Figure 3.4 A). However, wt and ND4 mice did not show significant differences in their expression of REF (Figure 3.4 A). Endogenous, intact REF from wt and ND4 mice neurons was purified by FPLC and subjected to linear-mode mass spectrometry (solid-state ionization) for comparison. REF derived from wt mice showed two major peaks (m/z 23730.25, 23737.41), while ND4 REF showed only one major peak (m/z 23730.28) (Figure 3.4B). Unmodified REF was expected to have m/z 23730, which is consistent with the observed peaks at m/z 23730.25 and 23730.28. The peak at m/z 23737.41 from the wt represents 7 deimination sites (Figure 3.4B), which was consistent with our amino acid analysis of REF (Enriquez-Algeciras et al., unpublished data; see accompanying manuscript). REF may possess additional modifications (methylation, myristolation) that either do not appear in the FPLC-
purified REF fractions or may occur too infrequently to be visible when compared to major peaks (non-modified and deiminated peaks).

3.2.4 Deficient ATP5b mRNA transport in ND4. Using qRT-PCR, we next determined whether ND4 and wt mice show differences in ATP5b mRNA transport (Figure 3.4 C). Although the total ATP5b mRNA concentration in the cytosol (Figure 3.4 C-a’) was highly similar, mt surface-localized ATP5b mRNA was decreased in ND4 mice relative to the controls (Figure 3.4 C-b’). This was indicative of normal ATP5b mRNA synthesis in the presence of impaired ATP5b transport. The relative levels of actin-related protein (ARP) mRNA, whether cytosolic or mitochondrial, was consistent (Figure 3.4 C-a” or Figure 3.4 C-b”’) between CD1 and ND4 mice. In order to determine whether there was a loss of mt surface-localized ATP5b mRNA in ND4 mice resulting from the loss of REF deimination, we carried out an IP experiment using anti-REF to determine the REF-bound mRNA fraction, comparing the ATP5b mRNA between ND4 and control mt. The mt fractions were prepared using a similar protocol to that used for qRT-PCR analysis (Figure 3.4 D). The anti-REF IP from the ND4 retinal mt fraction contained less ATP5b relative to controls, suggesting a decreased association between mRNA and REF in ND4 mice (Figure 3.4 D). This was most likely due to the loss of REF deimination. To confirm these findings, we also determined steady-state levels of ATP5b, which indicated that the levels of mt ATP5b were significantly lower in ND4 mice as compared to CD1 mice (Figure 3.4C-b’). This suggested that the ATP5b transport pathway may be impaired in ND4 mice.
3.2.5 Decreased ATP synthesis efficiency in ND4 mice. Mt respiratory defects have been proposed to occur in MS (Dutta et al., 2006). We investigated the function of the mt respiratory chain in ND4 and control mice by measuring the oxidation rate of different substrates in mitochondria that were isolated from mouse brain and spinal tissue. Our attempts revealed that mt respiratory defects were restricted to ND4 mice when compared to wt controls (Figure 3.6). The ADP-O (adenosine diphosphate-oxygen) ratio was also lower in ND4 mt compared to the wt control and corroborated the severity of synaptic mt defects resulting from the respiratory defects (Figure 3.7A and Table 3.2). Activity measurements of the respiratory complexes did not show a significant difference between CD1 and ND4 mice. However, enzymatic analysis of ATP synthase activity in the ND4 mice indicated that a defect was present (Figure 3.7B), and our overall results demonstrated decreased mt respiratory chain efficiency. It is possible that the lower ATP synthase activity observed in ND4 mice might be due to improper assembly of this respiratory chain complex. Thus, we used blue native denaturing gel (BN-PAGE) electrophoresis to address whether the respiratory complexes show decreased relative abundance or simply fail to assemble (Figure 3.5 A). The ratio of ATP5b levels in the assembled versus non-assembled fractions was found to be identical in mitochondria isolated from either CD1 or ND4 mice (Figure 3.5 A). These results suggest that the observation of lower complex V activity is due to lower levels of ATP5b (Figure 3.5 B) rather than impaired complex V assembly.
3.2.6 Stronger ATP5b-REF association in deiminated REF. We also attempted to determine the relative RNA binding ability of deiminated (d+) and non-deiminated REF using microarray analysis (GEO accession number GSE11843). Comparatively, deiminated REF showed stronger binding and higher enrichment for several mRNAs. The relative binding affinity of REF was determined by electrophoretic mobility shift assay (EMSA) (Figure 3.7C). Purified recombinant REF was prepared and analyzed in its non-deiminated form. Deiminated REF was prepared in vitro by incubating a fraction of the recombinant REF protein with purified PAD2 in the presence of calcium ions. Equal amounts of non-deiminated and deiminated REF were separately incubated with 0.5 nmol of ATP5b probe. Both reactions reached equilibrium within 10 minutes (Figure 3.7D). The time course comparison between deiminated REF and non-deiminated REF revealed that deiminated REF had a higher affinity to the ATP5b probe (Figure 3.7D). Calculations based on EMSA showed a greater dissociation constant for ATP5b mRNA and non-deiminated REF compared with deiminated REF; that is, 0.28 nM versus 0.15 nM under our test conditions, suggesting stronger binding between ATP5b mRNA and the deiminated protein.

3.2.7 Decreased deimination and reduced ATP synthase activity. In order to test whether deimination plays a role in other neuronal cell types, we used PC12 cells derived from rat pheochromocytoma (Greene and Tischler, 1976) and widely used as a neuronal cell line (Bischof et al., 2011). We transfected these cells with siRNAs against PAD2 and PAD4, the major deiminases in neuronal
cells, and determined the effect of subsequently-reduced deimination on cellular function (Figure 3.7E). The transfected cells demonstrated less neurite outgrowth (Figure 3.8 C), reduced PAD2 levels (Figure 3.7E), and reduced deimination of REF (Figure 3.7E bottom panel) relative to the controls. Additionally, ATP synthase activity in the transfected cells was significantly reduced (Figure 3.7F), which is consistent with our observation in ND4 mice (Figure 3.7 B).

3.2.8 The role of deimination in ATP5b mRNA transport. We also determined whether the deimination state of REF affected the association of ribosomal translation machinery components using in vitro purified REF or deiminated REF as bait (Figure 3.9A), and we found stronger association with deiminated REF than controls (Figure 3.9A). Based on these experiments, we hypothesized a model in which deiminated REF contributes to the transport of select mRNA (ATP5b, for example) to the mt surface (Figure 3.9B), thereby augmenting their translation. Accordingly, the siRNA knockdown of REF in primary hippocampal neurons resulted in reduced mt ATP5b levels (Figure 3.8A). Cytosolic ATP5b is also slightly reduced in these cells but not to the level of cytosolic or mitochondrial ATM1 (Figure 3.8A). We also found a reduction in mt ATPase activity (Figure 3.7F) in cells treated with REF siRNA compared to controls. A coupled transcription/translation system incorporating mt showed increased ATP5b accumulation in the presence of exogenously-added, in vitro deiminated REF but not recombinant, non-deiminated control REF (Figure 3.8C), suggesting a role for deiminated REF in ATP5b translation.
3.3 Discussion

MS is a neurodegenerative disease characterized by recurrent inflammation and the formation of localized, demyelinated plaques in central nervous system tissue (CNS; brain, spinal cord and optic nerve) and is associated with axonal neurodegeneration. Aberrant and simultaneous occurrence of hypo- and hyper-deimination has been observed in retinas from human MS patients and ND4 mice (as chapter II) The retinal ganglion layer shows hypo-deimination in contrast to hyper-deimination in other retinal cell layers, suggesting differences in the regulation of deimination activities in different cell types. Loss of deimination in the retinal ganglion cell (RGC) layer is evident, likely due to the absence of astroglial cells within this layer (Bhattacharya, 2009). We reasoned that this would extend to other generic neurons. The consequences of reduced deimination are likely to manifest as physiological symptoms of a disease state. Due to the heterogeneous nature of MS, different animal models will likely prove insightful toward a better understanding of the disease etiology. MS is known to be associated with mitochondrial (mt) respiratory defects (Dutta et al., 2006), which have been suggested to play an important role in axonal degeneration in MS (Campbell et al., 2010). Studies involving EAE (experimental autoimmune encephalomyelitis), a mouse model of MS, showed that mt defects occur early in disease development (Kaneko et al., 2006; Wujek et al., 2002), and when the mt defects were rescued, the axons were protected from severe damage in vivo (Chacko et al., 2010). Our attempts revealed mt respiratory defects in ND4 mice (Figure 3.6), and based upon respiration rates of mt respiratory chain complexes
I, III and IV in the presence of pyruvate + malate, we found a marginal decrease in state 3 respiration rates under most experimental conditions (Figure 3.7A, Table 3.2). Our results thus demonstrate marginally-decreased efficacy of complexes I, III and IV and suggest that the synergism of these three complexes is not affected by ATP5b (a part of complex V). Many mechanisms may contribute to decreased oxidative phosphorylation, including a general decrease in mt translation and a lack of proper ATP synthase assembly. No significant difference was observed in the efficiency of ATP5b component assembly between CD1 and ND4 mice in comparison to a control using NADH dehydrogenase [ubiquinone] iron-sulfur protein 4 (NDUSF4) (Figure 3.5 A). Mt activities are regulated at different stages during transcription, mRNA targeting to the mt, and assembly of the translated components within the mt. The process of mt protein import has now received wide attention (Chacinska et al., 2009; Neupert and Herrmann, 2007; Schnell and Hebert, 2003; Wickner and Schekman, 2005). However, the aspect of how pre-formed, nuclear-encoded mRNAs are selected and targeted for subsequent mt import is still unclear. Studies of lower eukaryotes, for example in yeast, have shown that some proteins undergo co-translational import into mt via surface bound ribosomes (Fujiki and Verner, 1991; Fujiki and Verner, 1993). This suggests the simultaneous existence of another transport mechanism that targets mRNA to the mt surface and facilitates local translation and import. In this process, the efficiency and accuracy of mRNA transport directly affects mt biogenesis. The
mRNA binding factors that associate with specific mt mRNA play an important role, and their mechanisms need to be more extensively studied.

Human REF, also called THO complex 4, Aly and BEF (bZIP enhancing factors), was found to express in many tissues, including retinal and brain (Ding et al., 2009; Levesque et al., 2001). Our initial studies showed that REF undergoes loss of deimination in ND4 mice compared to normal controls. Determination of mRNA binding by immunoprecipitation (IP) with deiminated and non-deiminated REF indicated that certain mRNA species were enriched by deiminated REF \(\text{[Table 3.1, GEO accession number GSE11843]}\). Enrichment of ATP5b, ATM1 and actin mRNA supports the assumption that mRNA export is the main function of REF in the cytosol \(\text{(Figure 3.1B)}\). A greater association of REF for ATP5b mRNA compared to that for ATM1 at the mt surface suggests that ATM1 and ATP5b are transported by two different pathways and that REF is likely involved in the ATP5b transport pathway \(\text{(Figure 3.1B)}\). Comparison of mt protein extracts from ND4 and wt control retinas showed loss of REF deimination \(\text{(Figure 3.4 A, B)}\) and ATP5b transport at the mt surface \(\text{(Figure 3.1 B and Figure 3.4C, D)}\) in ND4 mice relative to controls. Lower levels of ATP5b at the mt surface of ND4 mice compared to control CD1 mice \(\text{(Figure 3.5B)}\) may have been a consequence of mutation in the ATP5b structural sequence (Garcia et al., 2010), but restriction fragment length polymorphism (RFLP) mapping and sequencing did not show any difference in the ATP5b gene or transcript between ND4 and control CD1 mice. Binding affinity of the ATP5b mRNA by the deiminated REF is greater than that of non-deiminated REF, supporting an active
role for deiminated REF in ATP5b mRNA transport (Figure 3.7C, D). Furthermore, deiminated REF strongly interacts with protein translational machinery compared to control REF (Figure 3.9A). Based on these experimental results, we propose an active role for deiminated REF in the transport of select mRNAs on the mt surface (Figure 3.9B). Organelle localization of mRNA is influenced by several factors such as codon bias, 3’ UTR sequence, secondary structural elements in the mRNA, phylogenetic origin, and the length of the genes. Sylvestre and colleagues have calculated a synthetic localization value called the membrane versus free index (MFI) for mt localization of mRNA (Sylvestre et al., 2003b). In most cases, long mRNA prefer to localize to the vicinity of mt. What enables REF to contribute to ATP5b mRNA localization on the mt outer surface is not completely understood. ATP5b has a short 3’UTR (269 bases) compared to ATM1 (3479 bases), so ATP5b falls in to MFI class 1 (Sylvestre et al., 2003b). The 3’UTR sequence in combination with other REF co-binding protein factors may confer binding and localization specificities. Our current work demonstrates that deiminated REF plays a role in the transport of ATP5b mRNA on the mt surface, and future work will likely identify other elements that contribute to this process.

In summary, we demonstrate that ND4 mice present mt respiratory defects. Our results revealed a novel function of REF in mt mRNA transport, and we showed that decreased deimination of REF resulted in a decrease in ATP5b mRNA transport to the mt surface in generic neurons. We propose that mt health and biogenesis can be affected by a change in mRNA transport that results from
a loss of protein deimination. Future experiments will reveal whether or not this is likely to extend to other nuclear-encoded mt mRNAs for which REF may play a facilitating role in active transport. Cytosolic and mt protein extracts were prepared from ND4 and wt control mice retinas following established protocols. We performed immunoprecipitation (IP) to examine the presence of REF in mt and cytosolic fractions and observed its presence in both fractions (Figure 3.1A), as well as its ability to associate with different mRNAs. We identified a number of nuclear-encoded mt mRNAs, especially a series of ATP synthase complex mRNAs, which showed significantly increased binding with deiminated REF compared to control REF (Table 3.1, GEO accession number GSE11843). Mt mRNAs have been shown to be transported to the mt surface and locally translated (Corral-Debrinski, 2007; Corral-Debrinski et al., 2000; Kellems et al., 1975), including ATP2 and ATM1 in yeast (Corral-Debrinski et al., 2000). We sought to determine whether both ATP5b (mouse homolog of ATP2) and ATM1 bind to REF intracellularly, and the existence of ATP5b and ATM1 mRNAs at the mt surface were confirmed using reverse transcription PCR (Figure 3.1B-a’). IP of REF-associated protein/mRNA complexes was carried out with two different mitochondrial fractions derived from synaptic and non-synaptic environments, as well as with the cytosolic fraction. ATP5b and ATM1 mRNAs from the eluents were detected by PCR (as described above) (Figure 3.1B-b’), corresponding to the demonstration of ATP5b shown in both cytosolic and mitochondrial fractions (Figure 3.1B-b’). In contrast, actin mRNA (control) and ATM1 were detected only in the cytosolic but not in mitochondrial fractions (Figure 3.1B-b’). Detection
of actin and ATM1 in cytosolic fractions supports the mRNA export function of REF in the cytosol, where REF was expected to associate with most mRNAs. However, our PCR analyses revealed a stronger association between REF and ATP5b compared to that of ATM1 mRNA at the mt surface, suggesting that ATM1 and ATP5b may be transported by two different pathways. These results suggest that REF is involved in the transport of ATP5b.
Figure 3.1 REF and ATP5b status on mitochondrial fraction. (A) Demonstration of REF in mitochondrial fraction. Anti-REF IP from cytosolic or mitochondrial fraction. (a’) The input retinal cytosolic and brain mitochondrial protein extracts were separated on a 10% SDS-PAGE and detected with anti-COX IV and GAPDH as indicated. (b’) IP products from inputs as shown in (a’) were gel fractionated and visualized using silver staining. Bottom panel shows recovery of REF using Western analysis. (c’) Control IP with Aldehyde dehydrogenase 1 (ALDH1-IgG) antibody, bottom panels show Western blot detection with ALDH1 and REF antibody as indicated. (B) Detection of ATP5b and ATM1 mRNA by Reverse transcription-PCR (RT-PCR). (a’) ATM1 and ATP5b with wild type (WT) and ND4 mitochondrial (mt) fraction. (b’) Detection of ATP5b, ATM and actin in normal mouse cDNA derived from three IP products: Retinal cytosolic (cyt), non synaptic (mt-NS) and synaptic (mt-S) mitochondria as indicated were carried out using appropriate primer pairs. Control represents RT-PCR from an IP performed with an antibody unrelated to any known mammalian protein. Bottom panel shows amplification of actin used as a control. The product size has been provided in the parentheses.
Figure 3.2 Localization of REF on the mitochondrial surface. Primary hippocampal neuron culture was detected by (A, A’) Tomm20 and Porin antibody, (B, B’) REF antibody. Merged figure is shown as (C, C’). White boxes indicated co-localization of REF and Tomm20. Z axis view and magnified indicated by yellow arrows. Bar= 20µm. (D) Electron microscopy in detection of REF at mitochondrial surface.
**Figure 3.3 Electron microscopy in detection of REF at mitochondrial surface.**

Electron microscopic image of CD1 (A) and ND4 (B) mice optic nerve as indicated with no primary antibody (negative control) incubated with 10nm gold particle coupled secondary anti-mouse antibody. CD1(C) and ND4 (D) mice optic nerve as indicated with REF (mouse) primary antibody incubated with 10nm gold particle coupled secondary anti-mouse antibody. Bar= 0.2µm. Arrow and arrow head indicate the region shown in the inset and a mitochondrial gold particle respectively.
Figure 3.4 Comparison of ATP5b expression WT (CD1) and ND4 mice. (A) Loss of REF deimination in ND4 mice. Western analysis of deimination and REF in the cytosol and mitochondria of wild type (WT) and ND4 mice as indicated. The deimination status of REF was detected using anti-citrulline antibody (bottom panel), loading amount indicated by Porin. (B) Analysis of intact purified REF using mass spectrometry on a MALDI-TOF device in linear mode. Arrows head indicated non-deiminated form, arrows indicated deiminated form with m/z ratios. Cyt=Retinal cytosolic soluble proteins; mt=mitochondria IP- immunoprecipitation (C) ATP5b transcript levels were quantified and normalized by ATM1 mRNA levels by real time reverse transcription-PCR and compared between control (WT) and ND4 mice as indicated. (a', a") ATP5b and ARP mRNA levels in retinal cytosolic fraction and (b', b") mitochondrial fraction as indicated. ARP mitochondrial level is approximately ~2000 fold less compared to ATP5b or cytosolic ARP levels. (D) Comparison of REF and ATP5b association between WT (CD1) and ND4 mice. ATP5b expression from cDNA recovered by reverse transcription from IP products: wild type mitochondria (WT mt) and ND4 mitochondria (ND4 mt) as indicated was quantified by real time PCR and normalized by ATM1 levels. NS=non-synaptic mitochondria S=synaptic mitochondria. All comparisons were made from at least three independent measurements with analysis of variance expressed as mean ± the standard deviation (SD). All data were subjected to two-tailed paired t-test against controls (and were considered significant * p≤0.05).
Figure 3.5 Comparison of ATP5b expression between WT (CD1) and ND4 mice. (A) Blue native gel electrophoresis of mitochondrial protein extract from CD1 and ND4 mice as indicated (upper panel). Bottom panel shows Western blot analyses from a posttransfer 2D SDS-PAGE. Arrow head indicate NDUSF4; Proteins were probed with antibodies as indicated. (B) Western blot analyses of mt proteins from CD1 and ND4 mice with ATP5b and Porin as indicated.
Figure 3.6 Oxidation by mitochondria from WT (CD1) and ND4 mice brain and spinal cord in presence of 0.1 mM ADP. Oxygen uptake was measured polarographically at 30°C and pH 7.4 in a 0.3 ml reaction system. The non-synaptic and synaptic mitochondrial respiration is depicted by dashed and sold lines respectively. (A) Representative respiration rate profiles in mitochondria isolated from CD1 and (B) from ND4 brains. Solid line indicates non-synaptic mitochondria, dashed line indicates synaptic mitochondria.
Figure 3.7 Deiminated REF in regulation of ATP synthase expression. (A) Comparison of mean mitochondrial state 3 and 4 respiration rates between WT (CD1) and ND4 mice. Respiration rates (state 3 and 4 as indicated) in synaptic (S) mitochondria (a') and nonsynaptic (NS) mitochondria (b') isolated from ND4 (n= 4) and CD1 (n= 6) brains. Rate of oxygen consumption was measured in the presence and absence of ADP. (B) Comparison of mean mitochondrial ATP synthase activity between WT (CD1) and ND4 mice. Isolated mitochondria from brain nonsynaptic (solid column) and synaptosomes (hollow column). (C) Electrophoretic mobility shift assay (EMSA) analysis of deiminated REF using an oligonucleotide probe. EMSA of non-deiminated (d-) and deiminated (d+) REF as indicated in lane 2 and 3; the control lane was loaded only with probe. (D) Time course was generated by the ratio of bound and free probe (Bound/Free oligonucleotide) and incubation time and compared between non-deiminated REF (d-; cross and dashed line) and deiminated REF (d+; square; solid line). Protein loading from an EMSA gel was probed for REF and its deimination status (lower panel) using anti-REF and anti-citrulline with secondary anti-mouse and anti-rabbit coupled with IR-700 and IR-800 scanned on an Odyssey infrared imaging system. (E) PAD2 and GAPDH protein as indicated were compared between the control and PAD siRNA transfected cells. Bottom panel is probed with anti-REF and anti-citrulline antibody respectively. (F) The ATP synthase activity of PC 12 cell line after PAD2 and PAD4 siRNA treatment. Control=nonspecific negative siRNA, + siRNAs= PAD2 and PAD4 siRNAs. In all experiments above, variance are expressed by mean ± SD from at least three independent measurements. All data were subjected to two-tailed paired or unpaired t-test with respect to controls (*), *P≤0.05.
Figure 3.8 Comparison of neurites outgrow and ATP synthase activity after PAD2 and PAD4 siRNA treatment  Tomm20 (Cy5) and deimination (FITC) are detected on the PC12 cell after siRNA transfection. Merged figures have been shown as (A) PC 12 cells transfected with control siRNAs unrelated to any known mammalian, (B) PC 12 cells transfected with PAD2 and PAD4 siRNAs. Deimination levels were shown as (A’) control PC 12 cell, (B’) PC 12 cells transfected with PADs and PAD4 siRNAs. Arrow indicates neurite like projections. Bar= 10µm. (C) Comparison of neurites length between control and PADs siRNA treated PC 12 cell as indicated. Neurites length of PC 12 cells were measured from 5 different slides (2 neurons of each) using LAS AF (Leica Inc.) software. Variance is expressed as mean ± SD from a sum total of10 different neurites. This data was subjected to two-tailed unpaired t-test with respect to controls (*), *P≤0.05.
Figure 3.9 Association of deiminated REF with protein translational machinery components. Western analyses of from anti-REF (Refbp2) immunoprecipitation products with deiminated REF (d+) and control non-deiminated REF (d-) as bait (B). Schematic diagram of REF mediate mRNA export at mitochondrial surface. The REF (indicated) exports polyadenylated cargo from the nucleus. REF becomes additionally deiminated (indicated as d+) by PAD2 and recruits members of protein translation initiation complex elf4F, facilitate the mRNA (ATP5b) transport to mitochondrial surface; details of REF interacting protein-complex have been shown in the enlarged box.
Table 3.1 Fold increase in mRNA species bound with deiminated (d+) REF compared to non-deiminated (d-)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>REF (d+/d-)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atp5g2</td>
<td>2.2627</td>
</tr>
<tr>
<td>Atp6v1h</td>
<td>1.9814</td>
</tr>
<tr>
<td>Atp2b2</td>
<td>1.9749</td>
</tr>
<tr>
<td>Top1mt</td>
<td>1.987</td>
</tr>
<tr>
<td>Mrps21</td>
<td>1.9675</td>
</tr>
<tr>
<td>Mrps23</td>
<td>1.9592</td>
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<tr>
<td>Immp2l</td>
<td>1.9111</td>
</tr>
<tr>
<td>Mrpl17</td>
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</tr>
<tr>
<td>Mrps24</td>
<td>1.759</td>
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<tr>
<td>Atp5c1</td>
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<tr>
<td>Atp5o</td>
<td>1.6684</td>
</tr>
<tr>
<td>Tk2</td>
<td>1.6574</td>
</tr>
<tr>
<td>Clic4</td>
<td>1.6061</td>
</tr>
<tr>
<td>Timm23</td>
<td>1.5765</td>
</tr>
<tr>
<td>Atp5j2</td>
<td>1.5713</td>
</tr>
</tbody>
</table>

* Based on Microarray Analyses (GEO accession number GSE11843)
Table 3. 2 ADP-O ratios

<table>
<thead>
<tr>
<th>Samples</th>
<th>ADP/O ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 brain-NS*</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>CD1 brain-S</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>CD1 spine-NS</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>CD1 spine-S</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>ND4 brain-NS</td>
<td>2 ± 0.9</td>
</tr>
<tr>
<td>ND4 brain-S</td>
<td>2.1 ± 1.5</td>
</tr>
<tr>
<td>ND4 spine-NS</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>ND4 spine-S</td>
<td>1.6 ± 0.9</td>
</tr>
</tbody>
</table>

* NS: Nonsynaptic; S: Synaptic n=9 in each group

Table 3.3 Primers for different PCR experiments

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward Primer</th>
<th>Reverse primer</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP5b</td>
<td>CAGGCTATCTATGTGCTGATGAC</td>
<td>GCTTCTTTCAATGGGTCCCACCAT</td>
<td>*RT-PCR</td>
</tr>
<tr>
<td>ATM1</td>
<td>AGAAAGTGGCCATTGTAGGAGGTA GTG</td>
<td>GATTTTCATCTGATCAACCACTGTGA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>ATP5b</td>
<td>AGTTGCTGAGGTCTTCAGGG</td>
<td>CTTGCCACGGCTTTCTTC</td>
<td>**Q-PCR</td>
</tr>
<tr>
<td>ATM1</td>
<td>GGACTCCACACAGACCAA</td>
<td>CTGTTGAGGCTTCTACAGC</td>
<td>Q-PCR</td>
</tr>
<tr>
<td>PAD2</td>
<td>CCTACACA AGTTCT TGGAGA</td>
<td>GACAAGCGA GTCTACGGT TAGC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCACAGTCAAGGGCCGAAGAAT</td>
<td>GCCCTCTCCATGGGTGGTGA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Probe</td>
<td>TGGGCAGAATCATGAATGTC</td>
<td>EMSA</td>
<td></td>
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</tbody>
</table>

*RT-PCR=Reverse transcription PCR
**Q-PCR=Quantitative real time PCR
CHAPTER IV: Methods and materials

4.1 Mouse model, tissue procurement, general fixation, and immunohistochemistry. The original transgenic ND4 mice (Johnson et al., 1995; Mastronardi et al., 1993) were procured from The Hospital for Sick Children (Toronto, Canada) as a research gift. Animals were subjected to re-derivation using the services of Charles River Laboratory (Wilmington, MA). After confirmation of the genetic status, a colony was maintained at the Bascom Palmer Eye Institute. All animals were maintained according to Institutional Animal Care and Use Committee (IACUC) guidelines. Human donor MS tissue (brain and eye) was procured from the Human Brain and Spinal Fluid Resource Center (Los Angeles, CA). Normal and glaucomatous eyes were procured from the Florida Lions Eye Bank (Miami, FL) and the National Disease Research Interchange (Philadelphia, PA). Details of the donors are provided in Table 2.1.

Mouse tissues were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), 10% formalin alone, or in 4% paraformaldehyde, 0.2% glutaraldehyde in cacodylate buffer immediately after euthanasia. Human tissues were fixed in 4% paraformaldehyde in PBS within 8-24 hours after death. Immunohistochemistry was performed following established procedures (Bhattacharya et al., 2008), and a kit was utilized for immunohistochemical detection of protein-bound citrulline (17-347; Millipore Corporation). In each eye, three different but equivalent regions of fixed area were counted. Results from 5 eyes were presented as the average with associated standard deviations. All animal experiments were performed under an IACUC-approved protocol. All
human samples were handled in accordance with the tenets of the Declaration of Helsinki. All experiments with the human samples were conducted in the SKB Laboratory following procedures approved by the Institute Review Board (IRB) of the University of Miami. The cadaveric donor tissue was not regarded as human subject by the IRB, in accordance with NIH guidelines, and was exempted from the requirement of informed consent. Otherwise, informed consent was obtained from participants or their guardians where appropriate.

4.2 Confocal and immunofluorescence microscopy. Paraffin embedded sections (8 µm) from human and mice eye tissue were subjected to immunohistochemical analysis and imaged on a Leica laser scanning confocal microscope (TCS-SP5, Leica, Exton, PA). Imaging antibodies included anti-Thy1 (catalog number SC-9163, rabbit, Santa Cruz Biotechnology), anti-MAP2 (catalog number AB5622, rabbit polyclonal, Millipore) (secondary antibody coupled with Alexa 594) and anti-citrulline (secondary antibody coupled with Alexa 488). DAPI was used for general nucleic acid staining. Area measurements of comparable regions in retinal sections and the determination of relative intensities were performed using the Leica Application Suite, Advanced Fluorescence, 1.7.0 Build 1240 software. Counting of cells was performed on equivalent cross-sectional areas using anti-citrulline and Thy1 (or MAP2) in equivalent regions of retinal ganglion cell (RGC) layers. Throughout this work all counting was performed by three independent observers in a masked fashion. Antibody against Syntaxin [STX01 (HPC-1)] was obtained from Abcam (catalog number ab3265), and antibodies for γ-synuclein, NeuN and all PADs were
procured as research gifts from Drs. Andrei Surguchov, Barbara Grimpe and Hidenari Takahara, respectively. For obtaining representative images, a series of 1 µm xy (en face) images through the z-plane were collected and summed for an image representing a three-dimensional projection of the entire 8 µm section. Confocal microscopic panels were composed using Adobe Photoshop version 5.5.

4.3 Determination of retinal ganglion cells/Validation of retinal ganglion cell identity. Mice were euthanized by decapitation under halothane anesthesia. Eyes were rapidly perforated with a 30 gauge needle and vitreally injected to overflowing with 0.1 ml fixative. They were subsequently enucleated and hemisected, and the posterior pole was fixed for 24 hours. The fixative consisted of 4% formaldehyde, 0.1% glutaraldehyde, 0.1 M cacodylate buffer, 1mM MgSO4, 3% sucrose pH 7.4. Eyes were processed for epoxy embedding as described previously (30) and serially sectioned at 200 nm onto 12-spot polytetrafluoroethylene (Teflon; DuPont, Wilmington, DE) -coated slides (Cel-Line; Erie Scientific Inc.). Antibody probing was performed with primary IgGs targeting various molecules, and visualization utilized silver-intensified 1.4 nm gold granules conjugated to goat anti-rabbit IgGs. Deiminated cells in the RGC layer were visualized with an anti-citrulline protocol selective for protein-bound citrulline (Millipore anti-citrulline kit 17-347), and small molecule signatures of ganglion cells were determined with anti-GABA YY100 and anti-glutamate E100 IgGs (Signature Immunologics; Figure 2.2).
4.4 Imaging small molecule signatures. The small molecule signatures of cells in the RGC layer were imaged by conventional bright-field imaging as mosaic tiles of 8-bit, 1388-pixel x 1036-line frames under a voltage-regulated tungsten halogen light. Images were captured with a Peltier-cooled camera (Fast 1394 Qicam; QImaging) and autotiled with a montaging system (Syncroscan; Synoptics Inc). Final tiling and multimodal registration were performed with ir-tweak, a multi-platform registration application based on a Thin Plate Spline transformation. More information about ir-tweak and other related microscopy image processing applications can be found at http://www.sci.utah.edu/~koshevoy/research/.

4.5 Cloning, purification, RNA isolation and binding experiments. An REFBP2 clone (EMM1002-96824126) in pExpress1 was procured from Open Biosystems and subcloned in pQE1 vector (Qiagen) using an EcoRI and Xhol restriction digest and a primer set with the following sequences: 5′-CGTGGAGCCGAATTCATGGCCGACAAGATGGACATG-3′ and 5′-CACCACCCGCTCGAGGCTGGTGTTCCCTCCTTGCATTG-3′ (underline represents restriction sites). This clone was sequenced, transformed in E. coli M15 cells, and induced using 0.1 mM IPTG at OD$_{600}$ between 0.55-0.8. Recombinant 6xHis-tagged REFBP2 was purified using a two-pass method on a Ni-NTA column (Qiagen). Briefly, the cell lysate was prepared by sonication and centrifuged at 12000 x g for 15 minutes at 4°C followed by binding of the clear, soluble proteins onto a Ni-NTA column in PBS. The column was washed with 50 volumes of PBS, and recombinant REFBP2 was eluted with 100 and 250 mM
imidazole in the first pass. In the second pass, the purified product of first pass was extensively dialyzed using a 3500 MWCO membrane (Sigma Chemical Co.) in PBS and subjected to a second Ni-NTA purification, with eluent collection at 100 mM imidazole. The final purified product was again subjected to dialysis to render it free of imidazole. For *in vitro* deamination of recombinant REFBP2, a recombinant PAD2 was prepared. Briefly, the PAD2 clone (MHS1010-7507607) was procured from Open Biosystems and cloned into a pQE1 vector backbone as a GST (glutathione S-transferase) fusion gene using ligation of GST generated by PCR amplification. The GST column-purified PAD2 was incubated under deiminating conditions with dialyzed REFBP2, the product of the first Ni-NTA column pass described above. Recombinant REFBP2 (100 µg) was deiminated *in vitro* using 10 µg of recombinant PAD2 and subsequently repurified (second pass), dialyzed, and quantified using Bradford’s method.

For binding experiments, total RNA from 3 month old C57BL6/J mouse brain was isolated using TRIzol extraction according to standard protocols and was quality-verified by electrophoretic separation on a commercially-obtained gel (1.25% Seakem Gold Agarose in MOPS buffer, Latitude RNA precast gels, Cambrex Bioscience Rockland Inc.). RNA binding experiments were performed using 10 µg of purified 6xHis-tagged, recombinant, non-deiminated or deiminated REFBP2 incubated with about 400 µg total RNA. This mixture was subsequently incubated with 100 µl of Ni-NTA beads and loaded onto a mini column. The column was washed with 50 volumes of binding buffer, and bound protein was eluted in binding buffer containing 100 mM imidazole. The eluted RNA product
was precipitated using carrier BSA and subsequently chloroform-extracted and subjected to microarray analysis or separated on either a commercial gel or in-house-prepared composite 0.5% agarose-1% polyacrylamide gel. Quantitative (dissociation constant) electrophoretic mobility shift experiments utilized 10 µg recombinant or deiminated recombinant, 6xHis-tagged REFBP2 and varying amounts up to 0.5 mg of isolated, purified total RNA. The confirmatory qualitative experiments for bound RNA species (SNAP-25, VAMP2) were performed using total RNA derived from the retina and RGCs in a similar manner except for the use of 2 µg REFBP2 protein (recombinant and control) and 50 µg total RNA.

4.6 UV cross-linking experiments. UV cross-linking was performed using 0.5 µg of TUNP RNA binding protein (a research gift from Dr. Ralf Landgraf) and 6xHis-tagged, purified REFBP2 using total mRNA or 250 ng of SNAP25 mRNA purified using an antisense oligonucleotide (5′-ATGTCTGCGTCCTCGGCCAT-3′) column coupled to beads. Briefly, the RNA/protein mixture was incubated for 15 minutes at room temperature followed by exposure to 365 nm UV light for 45 seconds. The proteins were resolved on a denaturing 4-20% gradient gel and probed with radio-labeled oligonucleotides against SNAP25 mRNA. The gel bands were also excised, subjected to RNA extraction with a Qiagen kit, and PCR-amplified for a region of SNAP25 mRNA (Figure 2.3E).

4.7 Isolation and purification of REFBP2 from cells and tissues. REFBP2 was purified from isolated hippocampal neurons as well as retinal and brain tissues from wt mice. For purification from tissues, approximately 0.5 mg tissue samples were subjected to homogenization in 10 mM TrisCl pH 7.0, 50 mM NaCl
and 0.1% genapol following established procedures (Bhattacharya, 2006). The supernatant was centrifuged at 12000 x g for 15 minutes and then decanted in a fresh tube. The solution was precipitated with 20% ammonium sulfate, and the supernatant was loaded on an 8 cm Mono Q column (FPLC systems, GE Healthcare Inc.) and eluted with a gradient of 10-300 mM NaCl in 10 mM TrisCl pH 7.0. The REFBP2 eluted in fractions that corresponded to 200 mM NaCl based on conductivity measurements. The fractions were dialyzed and equilibrated to a buffer containing 10 mM NaCl, 10 mM TrisCl pH 7.0, loaded onto an 8 cm Mono S column, and eluted with a gradient of 0-300 mM NaCl. The REFBP2 was isolated in a fraction corresponding to 150 mM NaCl. Approximately 6 × 10^6 hippocampal neurons were used for the isolation of REFBP2. Approximately 1.2 × 10^7 neurons were subjected to nuclear and cytosolic fractionation for isolation of REFBP2 using a kit (Pierce Biotechnology Inc) and following accepted protocols (Picciani et al., 2009) that were similar to those used for other cell types in our laboratory.

4.8 Immunoprecipitation (IP). For IP, carefully dissected retinas from fresh mouse, pig, or human donor eyes (enucleated within 6-8 hours of death) were subjected to protein extraction. Approximately 100-200 μg of retinal extract or retinal cell extract in 10 mM TrisCl pH 7.0, 50 mM NaCl and 0.1% genapol was subjected to 2,3-butanedione monoxime and antipyrine treatment in an acidic environment using a citrulline kit (Millipore Corporation) for 10-30 minutes. This chemical modification added an adduct onto protein-bound citrulline residues that could then be recognized by an anti-citrulline-adduct antibody. Throughout this
work, we used this anti-citrulline-adduct antibody which did not recognize protein-bound citrulline unless the tissue or proteins were treated with 2,3-butanedione monoxime. The antibody also did not recognize free citrulline. Immediately after incubation, the acidic protein mixture was neutralized using 100 mM non-pH-adjusted Tris base solution and subjected to acetone precipitation at room temperature. Isolated RGCs obtained following the procedure described below were also used for anti-citrulline IP following 2,3-butanedione monoxime and antipyrine treatment in order to confirm that the identified retinal lysate proteins were RGC-derived. All IP experiments used null antibody (bead only) and non-specific antibody (anti-cochlin chicken and rabbit polyclonal) controls.

IP experiments for the identification of translation complexes were performed using 5 µg of recombinant, 6xHis-tagged, deiminated and non-deiminated REFBP2. Each batch was incubated with 1 mg of cytosolic retinal extract (nuclear and cytoplasmic extract kit, Pierce) for 1 hour, incubated with about 50 µl of Ni-NTA beads, and loaded onto a mini column. The column was then washed with 50 volumes of binding buffer (PBS and 5 mM imidazole), and the bound protein was eluted using both 100 mM and 250 mM imidazole solutions. Similar IPs were also performed with cultured RGCs and utilized 5 µg of REFBP2 (deiminated and control) and 100 µg of nuclear extracts. Eluted proteins were either dialyzed to remove imidazole or were acetone-precipitated (Patel et al., 2008) and subjected to further analysis.

For determination of eIF4B effects on REF-ribosomal complex formation, the product of anti-REF IP (250 µg) was subjected to subsequent IP with anti-
elF4B. The flow-through from the second column was recovered and passed through an oligo-dT column. An equal amount of SNAP 25 mRNA (250 ng) was transferred to 2 equal fractions of flow-through from the anti-elF4B column and incubated at room temperature for 10 minutes. To the separate fractions we added approximately 0.1 µg of purified, recombinant elF4B or CRALBP and subjected the fractions to a second IP with anti-REF. Purified, recombinant, 6xHis-tagged CRALBP was prepared from a construct following published protocols (CRALBP construct was a research gift from Dr. John Crabb). The PCR reaction for SNAP-25 has been described elsewhere in this text.

4.9 Other cell cultures and siRNA experiments. Two day old rat RGC neurons were used for siRNA experiments. These experiments were performed using siRNA against PAD2 and PAD4 sequences [Stealth Select RNAi for rat (Rattus norvegicus) PAD2 (catalog numbers, Oligo id#, RSS309706, RSS309707, RSS309705) and for PAD4 (catalog numbers, Oligo id#, RSS309711, RSS309713, RSS309712) from Invitrogen]. Control siRNA (Invitrogen) that had no homology to any known mammalian sequence was also used. The determination of neurite lengths for RGC neurons (and also generic neurons) followed published procedures with and without control siRNA treatment. Efficacy of PAD2 siRNA down-regulation was determined based upon previously-performed knockdowns on astrocytes (Bhattacharya, 2006). All siRNA used were carefully evaluated and found to down-regulate greater than 85% of PAD2 protein synthesis.
The mixed population of cells that were derived from carefully dissected RGC layers under a light microscope were stained for HPC1/syntaxin and citrulline as well as for RGC markers Thy1, γ-synuclein, or NeuN. Although we did not purify amacrine cells, only cells that were positive for RGC markers (Thy1, NeuN) were also positive for citrulline. Hippocampal neurons (embryonic E18) were prepared using established protocols (Ruthel and Banker, 1998). Briefly, after complete removal of the meninges and microdissection to remove the hippocampus, intact hippocampi were incubated in calcium-free Hibernate medium (BrainBits, LLC) containing 0.25% trypsin (Invitrogen) and 60 μg/mL DNase for 15 min at 37°C. The tissue was then rinsed extensively with Hibernate medium supplemented with 1X B-27 (Invitrogen), followed by trituration with a fire-polished Pasteur pipette to obtain hippocampal neurons. Retinal ganglion cell and hippocampal cells comprised the generic neurons used for the siRNA experiments described previously.

4.10 PC12 cell culture and siRNA treatment. PC12 cells were purchased from ATCC (Manassas, VA; ATCC# CRL-1721). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Manassas, VA) supplemented with 10% horse serum (ATCC), 5% fetal bovine serum (Cellgro), and 1% antibiotic-antimycotic solution (Cellgro). The siRNA experiments were performed using siRNA against PAD2 and PAD4 sequences [Stealth Select RNAi for rat (Rattus norvegicus) PAD2 (catalog numbers, Oligo id#, RSS309706, RSS309707, RSS309705) and PAD4 (catalog numbers, Oligo id#, RSS309711, RSS309713, RSS309712) from Invitrogen]. PAD2 and PAD4 are major cytosolic and nuclear
deiminases in the neuronal system that convert protein-bound arginines into citrullines. Control siRNA (Invitrogen) with no homology to any known mammalian sequence was also used. The siRNA was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, with minor modifications. Briefly, approximately 106 PC12 cells were collected for each transfection. All siRNA used were carefully evaluated and found to down-regulate approximately 50% of PAD2 and PAD4 mRNA expression. Approximately 106 siRNA (PAD2 and PAD4) -treated cells were used for each ATP synthase activity measurement. Cells were suspended in DMEM after transfection, plated onto 35 mm Petri-dishes coated with poly-D-lysine (Sigma Chemical Co., St. Louis, MO), and grown with differentiation medium containing 1% horse serum (ATCC), 1% antibiotic-antimycotic solution (Cellgro) and 100 ng/ml nerve growth factor (NGF) (Sigma Chemical Co.). The cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was exchanged every 3 days.

4.11 Detection of SNAP-25 and β-tubulin mRNA in laser-captured mouse neurites. Detection in mouse RGCs was performed via a non-radioactive, quantifiable detection system using probes directly labeled with horseradish peroxidase (HRP) or biotinylated probes. The SNAP-25 antisense oligonucleotide 5'-ATGTCTGCCTCCTCGGCCAT-3' (against Rattus norvegicus NM_011428) and the missense control oligonucleotide 5'-ATCTCAGCGTGCTTCGCCTT-3' as well as an additional control oligonucleotide (scrambled-sequence antisense oligo; 5'-TAGCTTCGGCTCGCTGCTGCTA-3')
were used as probes. For β-tubulin, a biotinylated or direct HRP-labeled probe (5’-TCTCGGCCTCGGTAACTC-3’) and an antisense control sequence (5’-AAGGCTTCCTGCACTGGTA-3’) against Mus musculus NM_023279 were utilized for quantitative estimations.

4.12 Amino acid analysis and linear-mode, solid-state ionization mass spectrometry. We performed amino acid analyses of purified, recombinant and non-recombinant, in vitro-deiminated REFBP2 as well as REFBP2 purified from nuclear and cytosolic fractions (from wt mouse; Table 2.3). About 25 µg of protein was subjected to overnight acid hydrolysis followed by analysis on a Hitachi L-8900 amino acid analyzer according to established procedures. The isolated REFBP2 protein from wt mice brains was also subjected to mass spectrometry on a MALDI-TOF mass spectrometer (Voyager DE Pro, ABI Inc.) in linear mode. This yielded a peak at m/z 23730.29 corresponding to unmodified REFBP2 and a large peak at m/z 23737.32 corresponding to the modification of 7 arginine residues. Comparatively lower-intensity ions were also recorded between m/z 23737.29 and m/z 23737.32, indicating the presence of intermediate modifications between 0 and 7 arginine residues. Endogenous REFBP2 from wt and ND4 hippocampal neurons was analyzed and compared using the same methods. Unmodified REFBP2 from wt neurons was observed at m/z 23730.25, while a large peak for m/z 23737.32 corresponded to deiminated REFBP2 (7 arginines were confirmed). REFBP2 from ND4 mice yielded only one peak at m/z 23730.28 that corresponded to unmodified REFBP2.
4.13 Mass spectrometric identification of deiminated peptides and modeling. REF (REFBP2) was initially purified from hippocampal neurons using an antibody column followed by Mono-Q and Mono-S columns on an FPLC system. REF protein solution (10 µM) was incubated with pepsin (sequencing grade; Princeton Separations) at 37°C for 3 hours in a 1:50 ratio of enzyme to substrate. After 3 hours, fresh pepsin was added, and this process was repeated for a total of three pepsin additions. The sample was then frozen until further analysis. Digestions were performed at pH 1.3 and pH 2.5. We also attempted the digestion of REF using other proteases (Chymotrypsin, AspN and LysC), but we found optimal results using the aforementioned protocol at pH 1.3. For peptide identification, 2 µL of pepsin-digested REF solution was analyzed using nano LC MS/MS (ESI) interfaced with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). The HPLC pump (1D Ultra, Eksigent) was configured for a “vent-load” nano-HPLC experiment, and the sample was injected across a self-packed 1 cm x 75 µm trap column (IntegraFrit, New Objective / Jupiter Proteo 4 µM, Phenomenex,) at 2 µL/minute (20 µL total). The vent was then closed, and the peptides were eluted across a self-packed 10 cm x 75 µm analytical column (IntegraFrit, New Objective / Jupiter Proteo 4 µM, Phenomenex,) at a flow rate of 275 nL/minute. Gradient elution was performed from 5% B (CH3CN, 0.1% FA) to 50% B over 120 minutes, and peptides were injected directly into the Orbitrap. MS/MS data were acquired in a data-dependent “top 5” experiment. Because the mass shift between unmodified and deiminated arginine was only 1 Da, the exclude width was reduced to 0.25 m/z.
The exclude list was limited to 500, and the exclusion duration was set to 30 seconds. MS/MS data were then searched with MASCOT (Matrix Science) and SEQUEST (Proteome Discoverer, Thermo Fisher Scientific) against a single protein database prepared from the Swiss-Prot database (plus a random decoy), and the results were merged into Scaffold (Proteome Software). The peptides identified from digestions performed at pH 1.3 provided 89.5% coverage, while those from pH 2.5 provided only 51% coverage. REF was modeled using PyMol from the NMR structure (accession number 2F3J) corresponding to SwissProt accession number Q9JJW6 (Figure 2.5).

4.14 Microarray experiments. RNA eluted from at least two independent binding experiments, each using recombinant and deiminated REFBP2, was subjected to cDNA preparation and two rounds of amplification using standard protocols. Four aliquots were subsequently hybridized (four independent hybridization assays) on an Affymetrix Mouse Genome 430 2.0 Array chip [NIH Neuroscience Microarray Consortium, Translational Genomics Research Institute (TGen), Neurogenomics Division], and the array hybridization results (abridged) were prepared (Table 2.2). The microarray data have been submitted to the GEO database, accession number GSE11843.

4.15 Culture of retinal ganglion cells, laser capture, and in vitro translation experiments. Rat RGCs were purified from a whole-retina cell suspension using established protocols (Barres et al., 1988). Briefly, rat retinal tissue was dissociated using papain, and an antibody-mediated plate adhesion (immuno-panning) procedure (Huettner and Baughman, 1986) was used to provide a
highly-pure population of ganglion cells. For retinas obtained from animals older than 3 weeks, a three-fold higher concentration of brain-derived neurotrophic factor (BDNF) was used.

The 5 day old retinal ganglion cells in culture showed neurite growth, so laser capture of the neurites was performed using Pixie III (Arcturus Inc.). Different batches of laser-captured neurites were maintained at -80°C prior to being pooled for preparation of the final extract. From the 225,000 laser captured neurites, protein extract was prepared and passed through a 3 mm microcapillary functionalized with oligo-dT. To the extract, 0.5 µg of SNAP-25 mRNA and a mixture of 20 amino acids were added to initiate translation with or without recombinant control and deiminated REFBP2. These experiments were performed following modifications to the previously published protocol for optic nerve extract (Bhattacharya et al., 2006b). As a control, an identical mixture without any REFBP2 and without the addition of SNAP-25 mRNA was also used. The translation mixture was then analyzed for accumulation of SNAP-25 protein. For this, the product of the *in vitro* translation mixture from each experiment was added in 20 µl aliquots to a 96-well plate (Costar 9018) and incubated for 20 min at room temperature. The supernatant was discarded, and the plate was washed with PBS. The plates were blocked with 1% BSA for 1 hour, washed with PBS, and incubated for 1 hour with goat polyclonal antibody to SNAP-25 (catalog number ab31281; Abcam, Inc.) The secondary antibody that had been coupled with alkaline phosphatase was then added and allowed to incubate for 1 hour, washed with PBS, and incubated with phosphatase substrate (100 µl/well) in
diethanolamine buffer pH 7.5, and the absorbance at 405 nm was measured on a
plate reader (BioTek SynergyHT). A similar experiment was also performed with
chicken polyclonal antibody to VAMP2 (catalog number ab14279; Abcam, Inc).

4.16 Virus production and transfection. The Thy1 promoter region falls 2000
bp upstream of the Thy1 start codon, and this sequence was identified using
UCSC Genome Bioinformatics database. A BAC clone with this region inserted
was obtained from Children's Hospital Oakland Research Institute (CHORI).
Primers (Forward 5′-AAAAACACGGTAGATCCAGAATGGGGGTG-3';
Reverse 5′-GTGGGGGCTAGCGGACAAAAGAAAAACTGCACAATA-3') including
restriction sites for Mlu1 and Nhe1 were prepared to amplify the region 0-2000bp
upstream of Thy1 start codon. PCR products were subjected to verification by
agarose gel electrophoresis. The PCR bands were excised and extracted using a
QIAGEN Gel Extraction Kit (Qiagen). The insert and vector (pLionII) were
simultaneously digested using restriction enzymes specific for the CMV region on
the vector, again subjected to gel electrophoresis, and extracted using the gel
extraction kit. The vector was dephosphorylated using calf intestinal alkaline
phosphatase (CIP). The digested vector and insert DNA were ligated in a ratio of
1:3 by incubating with T4 ligase at room temperature overnight. The ligated
constructs were used to transform competent TOP10 E. coli cells. Colonies of
transformants were picked, and the presence of the insert verified by double
restriction digestion of the plasmid. The construct was further confirmed by DNA
sequencing. The Thy1 containing pLionII was then restriction digested with
Pme1 and Not1. PAD2 was amplified using a clone vector (Open Biosystems)
with primers (Forward 5′-ATATAAGTTTAAAC ATGCTGCGCGAGCGGACCG-3′
Reverse 5′-TTTTGCGGCCGCTTACAGAGGAAAGCTGCTC-3′) containing the
Pme1 and Not1 restriction sites and was ligated into the Thy1 pLionII vector
using the same procedure as above. Thy1-PAD2 pLionII and CMV-YFP pLionII
constructs were separately transfected into human embryonic kidney cells (HEK-
293) using FuGENE 6 Transfection Reagent (Roche). Briefly, structural vector
(pCI-VSVG), envelope vector (pCPRΛEnv), and transfer vector (Thy1-PAD2
pLionII, empty pLionII vector with Thy1 promoter only or CMV-YFP pLionII) were
mixed in a 10:10:1 ratio. 21 µg of mixed DNA was incubated with 500 µl Opti-
MEM (GIBCO) containing 5 µl FuGENE and mixed with HEK-293 cells. Media
was changed every 3 days, and depleted media was collected and filtered using
a 0.4 µm Super Membrane (PALL). The filtrated media was mixed with 100%
PEG (Polyethylene Glycol 6000, USB Corporation) in a 6:4 (v/v) ratio and
centrifuged at 4°C for 20 min at 3500 rpm. The pellet was precipitated and then
resuspended in 300 µl DMEM. 10 µl of Thy1-PAD2 pLionII lentivirus was mixed
with 1 µl of CMV-YFP pLionII lentivirus, and the whole mixture was added to
HEK-293 cells. The neuronal cells were transfected using LipofactamineTM 2000
(Invitrogen).

4.17 Ocular Injections. The mice were anesthetized with intraperitoneal
ketamine (50 mg/kg) and xylazine (5 mg/kg). Subretinal injections were
performed under anesthesia following established methods using a 5 µl Hamilton
syringe connecting to an Ultra Micro Pump II (UMPII; World Precision Inc) to
deliver 0.5-1 µl of viral construct. Reproducible injections were achieved by the
UMPII device. An ointment containing antibiotics and gentamycin was applied to the injection site to prevent subsequent infection.

4.18 Flash Electroretinogram (FERG) and Pattern Electroretinogram (PERG) recording. FERG primarily reflected the function of our retinal neurons, whereas PERG primarily depended on the functional integrity of RGCs (Porciatti, 2007), and methods for FERG and PERG recording have been described previously (Porciatti et al., 2007). Briefly, ketamine/xylazine-anesthetized mice were gently restrained using a bite bar and a nose holder that allowed unobstructed vision and were kept at a constant body temperature of 37°C with a feedback-controlled heating pad. Eyes of anesthetized mice were typically wide open and steady, with undilated pupils pointing laterally and upward. The active electrode (0.25-mm diameter silver wire configured to a semicircular loop of 2-mm radius) was placed on the corneal surface by means of a micromanipulator and was positioned so as to encircle the pupil without limiting the field of view. Reference and ground electrodes were stainless steel needles inserted under the skin of the scalp and tail, respectively. A small drop of balanced saline topically applied on the cornea prevented drying for the duration of recording. For PERG recording, a visual stimulus of contrast-reversing bars (50° x 58° field area; 50 cd/m² mean luminance; 0.05 cyc/deg spatial frequency; 98% contrast; 1 Hz temporal frequency) was aligned with the projection of the pupil at a distance of 20 cm. Eyes were not refracted for the viewing distance given that the mouse eye has a large depth of focus because of the pinhole pupil. Retinal signals were amplified (10,000-fold) and bandpass filtered (1–30 Hz). Three consecutive responses to
each of 600 contrast reversals were recorded. The responses were superimposed to check for consistency and then averaged. PERG is a light-adapted response, so in order to have a corresponding index of outer retinal function, a light-adapted FERG was also recorded with undilated pupils in response to strobe flashes of 20 cd/m²/s superimposed on a steady background light of 12 cd/m² and presented within a Ganzfeld bowl. Averaged PERG and FERG readings were analyzed to evaluate the major positive and negative waves.

4.19 Isolation of polysomes and ribosomes. Polysomes were prepared from a total of $2 \times 10^6$ isolated RGCs that were washed with ice-cold isolation buffer R [20 mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl₂, 2 mM DTT containing protease mix (0.5 mg/ml Pefabloc SC, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 0.2% aprotinin, and 0.5 mg/ml heparin)]. Isolation buffer R containing 0.5% Igepal-600 (Sigma) was used to lyse RGCs, and cytoplasmic extracts were obtained after centrifugation at 25,000 × g for 15 min at 4°C. Cytoplasmic extracts so prepared were centrifuged at 100,000 × g for 3 hr at 4°C for generation of a polysome-enriched pellet (P100) and a post-polysomal supernatant (S100). For preparation of ribosomes, all steps were identical except that RGCs were suspended in isolation buffer R with protease mix and 100 μg/ml cycloheximide (Sigma Chemical Co.) to block the elongation step. Cytosolic extracts were prepared using 0.5% Igepal-600 and 100 μg/ml cycloheximide, and cytoplasmic extracts were obtained after centrifugation at 25,000 × g for 15 min at 4°C. The clear extract was loaded onto a linear 10–50% (w/v) sucrose gradient in isolation
buffer R and centrifuged at 100,000 \times g for 3 hr at 4°C. Gradients were fractionated by upward displacement with 70% sucrose, and the absorbance at 260 nm was continuously monitored. Ribosomal subunits (60S, 40S) were fractionated by resuspending the polysomes in 20 mM Tris pH 7.5, 500 mM KCl, 3 mM MgCl$_2$, 2 mM DTT and subjecting them to centrifugation at 100,000 \times g for 24 hr at 4°C.

Mouse or rat retinal polysomes and ribosomes were also prepared. Retinas from freshly euthanized animals were quickly removed and placed in ice-cold isolation buffer R containing 100 \mu g/ml cycloheximide. All subsequent operations were performed at 4°C. Tissues were lysed and homogenized in isolation buffer R using a hand-held Kontes homogenizer. A supernatant (post-mitochondrial fraction) was prepared by two consecutive centrifugations of the homogenate at 30,000 \times g for 15 min. To this supernatant, Triton X-100 was added to a concentration of 1%, and the supernatant was incubated for 15 min. Subsequently, the supernatant was layered onto a step gradient containing 7.5 ml of 0.7 M (w/v) sucrose on top of 8 ml of 1.6 M (w/v) sucrose (both in isolation buffer R) and centrifuged at 100,000 \times g for 18 h. The polysome-enriched pellet was resuspended in isolation buffer R containing 100 \mu g/ml cycloheximide and layered onto 10–50% sucrose gradients prepared in the same buffer. The gradients were centrifuged and fractionated as described above. To dissociate ribosomal subunits (60S and 40S) from rat tissues, polysome-enriched pellets were resuspended in 20 mM Tris pH 7.4, 500 mM KCl, 3 mM MgCl$_2$, 2 mM DTT, 1 mM puromycin (added to a final concentration of 1 mM). Samples were
incubated at 37°C for 15 min and centrifuged twice for 15 min at 30,000 × g, and the supernatant was loaded on a linear sucrose gradient (10–30%) in isolation buffer R. The gradients were centrifuged at 100,000 × g for 6 hr at 4°C, and fractions were collected by upward displacement with 70% sucrose as mentioned previously. Fractions corresponding to particular subunits were pooled, diluted with isolation buffer R, and subjected to centrifugation for 24 hr at 100,000 × g for precipitation. RNA was isolated from different fractions using established methods and then subjected to analysis.

4.20 Isolation of brain mitochondria. Brain mitochondria were isolated following a procedure previously described (Dunkley et al., 1988). Generally, mice were decapitated under isoflurane anesthesia, and the brains and spinal cords were removed immediately and immersed into cold (4°C) isolation medium. Isolation medium consisted of 250 mM sucrose, 1 mg/ml bovine serum albumin (BSA; EMD, Gibbstown, NJ), 1.0 mM EDTA, and 0.25 mM DTT pH 7.4. Tissue was minced with scissors and rinsed thoroughly with isolation medium. The minced tissue was suspended in isolation buffer and homogenized with a hand-operated Teflon homogenizer by 7 up-and-down strokes. The homogenate was diluted to a final concentration of 10% (w/v). The homogenate was centrifuged at 500 X g for 5 min in a Sorvall RC5 centrifuge. The supernatant was collected from 3 mice brains and spinal cords. The pooled supernatant was layered on Percoll gradients prepared in 12 ml polycarbonate tubes that consisted of 2 ml each of 23%, 15%, 10%, and 3% (v/v) Percoll. The gradients were centrifuged at 32,500 X g for 5 minutes. The non-synaptic mitochondrial pellet was collected.
from the bottom of the 23% Percoll layer, while synaptic mitochondria were collected at the interface between the 23% and 15% as well as the 15% and 10% layers. Both fractions were then combined. The collected mitochondria were washed once with isolation media and, using 12 ml polycarbonate tubes, were pelleted by centrifugation at 15,000 X g for 10 min. The resulting non-synaptic and synaptic mitochondrial pellets from different fractions were resuspended in 0.32 M sucrose to give concentrations of approximately 4-7 mg/ml.

4.21 Respiration studies in isolated mitochondria. Substrate oxidation rates and the phosphorylating capacities of isolated mitochondria were determined by polarography following a previously-described procedure (Dave et al., 2001; Hofhaus et al., 1996). Oxygen consumption was measured in 300 µl of 25 mM Tris–HCl, 10 mM potassium phosphate, 150 mM sucrose pH 7.4 using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England), which was positioned in a 0.25 mL-capacity microassay incubation chamber incorporating a water jacket (30°C). Inside the chamber, buffer was stirred continuously at 40 rpm using an electromagnetic bar stirrer. The oxygen electrode was connected to a computer, and oxygraphs were recorded using the manufacturer’s software (Hansatech Instruments). For respiration studies, synaptosomes were permeabilized with 0.007% digitonin.

The mt respiratory control index (RCI) and ADP–O ratios were measured in the presence of 5 mM pyruvate and 2.5 mM malate. For each experiment, approximately 0.03-0.05 mg mt protein was added to the assay. RCI is defined as the ratio of respiratory rate in the presence (state 3) and absence (state 4) of
ADP (Chance and Williams, 1956). ADP–O ratios were measured using polarography, as described previously (Chance and Williams, 1956). In brief, the ADP–O ratio was defined as moles of ADP phosphorylated per moles of oxygen consumed (slope of state 3 over the time required for total consumption of ADP added to the buffer).

4.22 RNA extraction and reverse transcription PCR. As described previously, anti-REF IP experiments were carried out using mt and retinal cytosolic extracts. The eluents were collected separately, and RNA species eluted together with REF and were isolated using a miniRNA extraction kit (Stratagene Inc., La Jolla, CA) as per the manufacturer’s recommended protocol. Total RNA was dissolved in DEPC-treated distilled water and converted to cDNA using oligo-dT (12-18 nt) (Invitrogen Inc., Carlsbad, CA) in two-step reactions as provided by the kit. For detection of mRNA species, IP product-derived mRNA was converted to cDNA via qRT-PCR using primers for Tfam, β-actin, ATP5b and ATM1 (Table 3.3). PCR reactions were performed under mild conditions as per the manufacturer’s instructions (Advantage cDNA PCR kit; Clontech Inc., Mountain View, CA), and the PCR product was separated on a 2% agarose gel in TBE buffer.

4.23 Real Time PCR. Quantitative real-time PCR was performed via an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA) running built-in interface software (version 2.3; Bio-Rad) using the primer pairs shown in Table 3.3. All PCR experiments were carried out in triplicate using a reaction volume of 25 μl in iCycler IQ 96-well optical-grade PCR plates (Bio-Rad) covered with iCycler
optical-quality sealing film (Bio-Rad). Mastermix was prepared as follows (to the indicated end-point concentration): 0.4 µM forward primer, 0.4 µM reverse primer, and 12.5 µl of SYBR Green PCR Universal master mix (2X, Bio-Rad). Amplifications were performed using the following temperature procedure: one initial denaturation cycle at 95°C (3 min), 35 denaturation cycles at 95°C (30 seconds), primer annealing at 56°C (30 seconds), and a final denaturation cycle at 95°C (30 seconds). Finally, melt curve analyses were made by slowly heating the PCR mixtures from 55 to 95°C (1°C per cycle of 10s) with simultaneous measurements of SYBR Green I signal intensities. Quantitation was performed using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers.

4.24 Electrophoretic Gel Mobility Shift. The non-radioactive LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Rockland, IL) and 5'-biotin end-labeled oligonucleotides were used according to the manufacturer’s recommended protocol for the detection of RNA and protein interactions. Recombinant, non-deiminated REF and in vitro-deiminated, recombinant REF were generated to test with different single-stranded DNA probes (Table 3.3). Purified REF protein (10 µg) was incubated with 0.25 pM single-stranded, biotin-labeled oligonucleotide, 20 mM KCl, 2 mM MgCl₂, 4 mM EDTA, 1% glycerol, 1 ng poly-dI-dC, and 0.025% NP-40 in binding buffer for a total volume of 20 µL at 37°C for 60 min. To determine binding specificity, supershift analysis was performed via the addition of 0.5 µg of anti-REF (Aviva Systems Biology, San Diego, CA) after a 60 min binding reaction and
then incubated for an additional 10 min at 37°C. Specific and nonspecific competitions were performed using non-biotinylated, specific-sequence oligonucleotide and poly-dl-dC, respectively (data not shown). The samples were then run on a 6% DNA retardation gel (Invitrogen) at 100 V for 60 min. Protein was electrophoretically transferred to a positively-charged nylon membrane (Pierce Biotechnology, Rockland, IL) at a constant current of 380 mA for 1 hour on ice, and the membrane was immediately UV cross-linked (254 nm) for 60 seconds at 120 mJ/cm² using a UV transilluminator. Streptavidin-horseradish peroxidase conjugate and LightShift chemiluminescent substrate were used to detect the biotin end-labeled DNA. Nylon membranes were then exposed to X-ray film for detection.

4.25 ATP synthase activity measurement. This assay was performed with 10-15 μg of mt protein at 37°C using 0.12 mM NADH as a donor with 9-14 unit/ml pyruvate kinase/lactic dehydrogenase enzymes (Sigma Chemical Co.), phosphoenolpyruvic acid (Sigma Chemical Co.), and 2 μg/ml antimycin in 100 mM Hepes-KOH buffer containing 10mM MgSO₄, pH 8.0. The baseline was recorded at 340 nm before adding 2.5 mM ATP, and the reaction was recorded at the same wavelength upon reaching linear phase. The reaction was recorded again after inhibition by 5 μg/ml oligomycin (Sigma Chemical Co.). ATP synthase activity was calculated using the Beer-Lambert equation (Lemaire and Dujardin, 2008; Rimoldi and DiDonato, 1982).

4.27 Electron Microscopy. Optic nerve tissues dissected from mice were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M phosphate buffer pH 7.5.
Optic nerves were dehydrated in a graded ethanol series (30%, 50% and 70%), transferred into LR White™ embedding resin, and subjected to overnight polymerization at 60°C. The embedded tissue was cut into 65 nm sections on nickel grids (200 mesh). Immunogold staining was performed by following previously-published methods with minor modifications (Roth, 1984). Briefly, nickel grids mounted with sections were floated for 1 hr on a drop of 50 mM Tris acetate pH 7.2, 250 mM sucrose, 0.5% BSA. The specimen grids were incubated in a solution of primary anti-REF antibodies (Abcam) that were diluted 1:200 in Tris-sucrose buffer as described above. Controls were tissues incubated in the same buffer without primary antibody. After 1 hr incubation at room temperature, the grids were rinsed with 9-12 drops of Tris-sucrose without BSA, followed by incubation for 1 hr at room temperature in goat anti-mouse IgG gold (10 nm, Amersham) that was diluted 1:40 in Tris-sucrose buffer. The grids were washed with Tris-sucrose buffer and distilled water. Sections were stained with uranyl acetate followed by lead citrate and examined with a Philips CM10 electron microscope (21,000X magnification).

4.28 Statistical analysis. Comparisons were made from at least three independent measurements, with analysis of variance expressed as mean ± standard deviation (SD) unless stated otherwise. All data were subjected to a two-tailed paired t-test against controls (p ≤ 0.05).
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