The Role of Lamin B1 in the Organization of the Nuclear Envelope and Myelin Regulation in Development and Disease

by

Bruce C. Nmezi

BS Biology, University of Pittsburgh, 2009

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This dissertation was presented

by

Bruce C. Nmezi

It was defended on

July 10, 2019

and approved by

Kris N. Dahl, PhD Professor of Chemical Engineering Carnegie Mellon University

Robert D. Nicholls, DPhil Professor of Pediatrics School of Medicine, University of Pittsburgh

Beth L. Roman, PhD Associate Professor of Human Genetics Graduate School of Public Health, University of Pittsburgh

Zsolt Urban, PhD Associate Professor of Human Genetics Graduate School of Public Health, University of Pittsburgh

Dissertation Advisor: Quasar S. Padiath, MBBS, PhD Associate Professor of Human Genetics, Graduate School of Public Health, University of Pittsburgh Copyright © by Bruce C. Nmezi

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Bruce Nmezi, PhD

University of Pittsburgh, 2019

Abstract

The nuclear lamina is a structural meshwork composed of intermediate filament proteins known as lamins that maintains nuclear shape and function. Perturbations of lamins lead to diseases, collectively known as laminopathies, which affect a wide variety of organ systems. One such laminopathy is autosomal dominant leukodystrophy (ADLD), a severe and fatal adult-onset demyelinating laminopathy caused by overexpression of lamin B1, one of the lamin proteins that make up the nuclear lamina. My studies aim to elucidate the role of lamin B1 in the organization of the nuclear envelope, its role in myelin regulation during oligodendrocyte maturation, and to understand how the genomic rearrangements involving LMNB1 cause ADLD. Our results suggest a novel concentric organization model of the nuclear lamina, with lamin B1 facing the inner nuclear membrane while lamins A and C together face the nucleoplasm. Lamin B1's outwardfacing localization maintains nuclear shape by restraining the lamin A/C meshwork from protruding outward. To study lamin B1's function in mature oligodendrocytes, conditional Lmnb1 knockout mice were used to study behavioral and molecular changes in the central nervous system. Knockout mice did not exhibit any overt behavioral phenotypes or myelination defects, but a careful analysis revealed alterations in the number of myelinating oligodendrocyte populations. We conclude that while mature oligodendrocytes do not require lamin B1 for their proper function, it might be important for the regulation of oligodendrocyte cell number. Array CGH studies

revealed that deletions upstream of *LMNB1* can also lead to ADLD, while large duplications involving *LMNB1* and a significant upstream region do not. Real-time PCR analysis demonstrate much higher *LMNB1* expression in white matter than in grey matter and fibroblasts. We propose that an oligodendrocyte-specific silencer element lies upstream of *LMNB1*, explaining ADLD's central nervous system exclusivity despite a constitutional *LMNB1* duplication. As demyelination and white matter injuries are common in disorders affecting a wide age range – from preterm neonates to young adults and the elderly – researching pathways involved in myelination and ways to reverse it could have a significant impact to public health.

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Preface

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List of Abbreviations

aCGH	Array-based Comparative Genomic Hybridization	
ADLD	Autosomal Dominant Leukodystrophy	
BSA	Bovine Serum Albumin	
Cal-A	Calyculin A	
cKO	Conditional Knockout	
CNS	Central Nervous System	
CNV	Copy Number Variation	
DMEM	Dulbecco's Minimal Essential Medium	
EdU	5-Ethynyl-2'-Deoxyuridine	
EM	Electron Microscopy	
FB	Forebrain	
FBS	Fetal Bovine Serum	
Flox	Flanked by LoxP sequences	
IHC/IF	Immunohistochemistry/Immunofluorescence	
INM	Inner Nuclear Membrane	
LA/C	Lamin A/C	
LB1	Lamin B1	
Lmnb1	Mouse gene for lamin B1	
LMNB1	Human gene for lamin B1	
MEF	Mouse Embryonic Fibroblasts	
OL	Oligodendrocyte	
OPC	Oligodendrocyte Progenitor Cell	
PBS	Phosphate Buffered Saline	
rtTA	Reverse Tetracycline Transactivator (Tet-ON)	
SC, SCC	Cervical Spinal Cord	
SNP	Single Nucleotide Polymorphism	
STORM	Stochastic Optical Reconstruction Microscopy	
TAD	Topologically-Associated Domain	
TRE	Tetracycline Response Element	
Wt	Wild-type	

1.0 Introduction

1.1 The Nuclear Lamina

1.1.1 The structure of the nuclear lamina

The nuclear lamina is a filamentous meshwork that lies beneath the inner nuclear membrane in all metazoan cells. It regulates the size, shape, and structural integrity of the nucleus (1). Type V intermediate filaments known as lamins constitute the nuclear lamina, of which two main types have been identified: A-type and B-type lamins. A-type lamins consist of lamins A and C, both of which are products of the *LMNA* gene in 1q21 through alternative splicing. B-type lamins consist of lamins B1 and B2, coded for by two different genes: *LMNB1* in 5q23 and *LMNB2* in 19q23, respectively (2). Structurally, lamins have much in common with other intermediate filaments: they are composed of α -helical central rods flanked by an N-terminal globular "head" and a C-terminal "tail" domain (3). The central rod domains dimerize in a coiled-coil fashion, in turn assembling in a head-to-tail manner and associating laterally in a half-staggered, anti-parallel fashion to form filamentous arrays (Figure 1.1) (4, 5). The nuclear lamina meshwork lies just beneath the inner nuclear membrane and can alter gene expression through chromatin interactions as well as affect the spacing of nuclear pore complexes (Figure 1.2) (6, 7).



Figure 1.1: Polymerization of lamin protein.

Lamin polypeptides dimerize at their central rod domains, which in turn associate in a head-to-tail manner. Polymers then associate laterally, in a half-staggered, antiparallel fashion to form filamentous arrays.



Figure 1.2: Location of the nuclear lamina.

The nuclear lamina is a meshwork of intermediate filaments within the nucleus, located underneath the inner nuclear membrane. It plays a role in chromatin organization.

1.1.2 Lamins in development

A-type lamins are primarily expressed in differentiated cells, with some notable absences in certain tissues. For example, while neurons and glia within the central nervous system (CNS) are high in lamin C expression, they contain little to no lamin A. *Lmna*-null mice develop normally for the first few weeks, however, they succumb to muscular dystrophy and cardiomyopathy after 4-6 weeks of age (8). In contrast, B-type lamins are expressed in all mammalian cells throughout development and differentiation, and *Lmnb1*-null mice die shortly after birth with skeletal and lung defects (9). Lmnb1-null studies must consequently be done in a tissue-specific or conditional knockout manner. Lmnbl is required for proper mouse organogenesis as well as neuronal migration during development (10, 11), but the absence of *Lmnb1* in mouse keratinocytes has no effect on the proliferation or development of skin and hair cells (12). In these tissues, A-type lamins are hypothesized to be sufficient for maintaining skin cell proliferation and vitality in vivo. These data together show that lamin B1 can be of variable importance in different tissue types during development. Previously, it has been hypothesized that A-type and B-type lamins may be able to operate as independent network systems within the nucleus, as opposed to simply being incorporated into one large, heteromultimeric meshwork along the inner nuclear membrane (13-15). Research on their spatial organization within the nuclear envelope was unclear until now: my dissertation will describe the various microscopic and biochemical methods utilized to elucidate nuclear lamina structure.

In vitro studies show that A-type and B-type lamins operate independently to regulate nuclear shape and gene expression. *Lmna*-knockout studies in mouse embryonic fibroblasts demonstrate abnormally elongated nuclei with the loss of nuclear envelope proteins including B-type lamins from one pole, through which herniations of chromatin may be seen (8). In contrast,

knockouts of *Lmnb1* result in excessive nuclear envelope protrusions known as blebs, which almost exclusively contain A-type lamins, and are highly associated with gene-rich euchromatin (13). Depletion of either lamin A or lamin B1 results in abnormal chromatin decondensation and loss of heterochromatin (16, 17), suggesting that A-type and B-type lamins play supportive roles in chromatin organization.

1.1.3 Post-translational processing of nuclear lamins

B-type lamins and prelamin A (the precursor to mature lamin A protein), have a C-terminal CAAX motif (C = cysteine, A = aliphatic, X = any amino acid), to which farnesyltransferase enzymes add a 15-carbon farnesyl group onto the cysteine residue. This in turn leads to endoproteolytic release of the last 3 amino acids, and finally carboxymethlation of the remaining farnesylcysteine residue (18, 19). Prelamin A is processed into mature lamin A by metallopeptidase ZMPSTE24, which catalyzes proteolytic cleavage of 15 amino acids at the C-terminus, losing the farnesyl moiety in the process (20-22). The C-terminal domains of B-type lamins are kept intact, remaining permanently farnesylated (Figure 1.3) (23). Lamin C, the shorter product of the *LMNA* gene through alternative splicing, is never farnesylated (24).

It is hypothesized that this farnesyl group allows the B-type lamins to form a tight anchor within the inner nuclear membrane, affecting its localization within the nucleoskeleton. Inhibition of B-type lamin farnesylation through farnesyltransferase inhibitors or a mutation in the CAAX domain leads to its aberrant localization, malformed nuclei, and chromatin migration deficiencies in neurons (23, 25, 26).



Figure 1.3: Processing of lamins A/C and B1 (simplified).

Lamin B1 precursor (green, left) has a 15-carbon farnesyl group attached to the C-terminal by farnesyltransferase enzyme (FTase), and the mature lamin B1 protein remains permanently farnesylated. Lamin A precursor (red, right) is also farnesylated, but the C-terminus is later cleaved by proteolytic enzyme ZMPSTE24. The mature form of lamin A lacks a C-terminal farnesyl group. Lamin C is also a product of the *LMNA* gene through alternative splicing, but is never farnesylated.

1.2 Myelin

White matter composes up to 40% of the mature human brain (27), of which myelin is the major component. Composed of sheaths made from many phospholipid bilayers wrapped around axons in the brain and spinal cord, its purpose is to enable saltatory transmissions of nerve impulses along the lengths of the neurons (Figure 1.4A and B). In the central nervous system (CNS), myelin is produced by a special type of cell known as oligodendrocytes and is a modification of oligodendrocyte cell membranes. High concentrations of myelin and oligodendrocytes can be found in the major white matter tracts of the central nervous system, such as the corpus callosum, which connects the left and right hemispheres of the brain and acts as a popular anatomical landmark due to its size and ease of identification.



Figure 1.4: Myelination and oligodendrocytes.

(A) Electron micrograph image of mouse corpus callosum shows cross sections of axons and their myelin sheaths. Scale bar: $1\mu m$. (B) Illustration of an axon surrounded by myelin wrapping. (C) A single mature oligodendrocyte (green) is capable of myelinating multiple axons.

1.2.1 Oligodendrocytes

Oligodendrocytes, along with astrocytes and microglia, comprise a group of cells known as glia. Glia compose the majority of cells in the nervous system, and they provide structural support, signaling, and safety for neurons (28). Oligodendrocytes themselves make up 20% of the cells in the brain (29), and are responsible for myelination in the CNS, as opposed to Schwann cells in the peripheral nervous system. Depending on the region of the CNS, one oligodendrocyte is capable of myelinating anywhere from a single axon to dozens of axons (Figure 1.4C) (30). They differentiate from multipotent cells known as oligodendrocyte progenitor cells (OPCs), which are in turn derived from the neural tube epithelium during early gestation (31). OPCs are defined by their ability to proliferate and migrate, but not myelinate, as well as their expression of surface markers such as platelet-derived growth factor receptor alpha (PDGFR α) and NG2 proteoglycan (32). Cessation of OPC proliferation and migration is necessary to differentiate into mature (a.k.a. myelinating) oligodendrocytes, but artificially arresting OPC proliferation is not sufficient to induce differentiation *in vivo* (33). Proper signaling pathway activation is required for successful differentiation, such as decreased Wnt/beta-catenin signaling (34), decreased Notch signaling (35), and increased ERK-1/2 levels (36). Epigenetics, non-coding RNAs, and external stimuli have also been implicated in regulating OPC differentiation (37-39).

1.2.2 Proteins involved in myelination

Axons in the nervous system are wrapped in extensions of the oligodendrocyte cell membrane known as myelin. In mice, myelination starts at birth in the spinal cord, and is generally completed at around two months of age (28). Myelination in the human CNS takes years and is generally completed during the second decade of life (31). Proper formation of myelin requires significant investment of lipids, proteins and enzymes, a few of which will be covered here.

Proteolipid protein 1 (PLP1), is a major component of myelin, a transmembrane protein that constitutes 50% of the myelin protein in the CNS (40). It most likely binds to other copies of itself spaced along the myelin membrane in order to aid folding, compaction, and maintenance of myelin's multilayered structure (41). It is coded by the *PLP1* gene on Xq22, predominantly expressed in oligodendrocyte lineage cells, and is highly conserved across humans, apes, and rodents. Mutations of *PLP1* are associated with dysmyelinating neurodegenerative disorders such as Pelizaeus-Merzbacher disease and spastic paraplegia type 2 (42, 43). Myelin basic protein (MBP), constituting 30% of the CNS myelin protein (40), is the second most common protein component of myelin. Coded for by the *MBP* gene in 18q23, MBP is highly cationic and assists with myelin wrapping by interacting with negatively-charged phospholipid groups as well as PLP1 in the membrane (40, 44). Absence of MBP in mice leads to severe CNS hypomyelination, resulting in a shivering phenotype and an early death (45, 46). Autoimmunity to MBP in humans is also linked to pathogenesis of multiple sclerosis (MS) (47).

Myelin oligodendrocyte glycoprotein (MOG) is a transmembrane adhesion molecule that is found exclusively in the CNS (48). It is hypothesized to homodimerize along the myelin sheath to maintain tight wrapping, similar to PLP (49). Autoimmunity to MOG antigens is also implicated in the onset of MS (49).

Myelin associated glycoprotein (MAG), another transmembrane glycoprotein, is found in both the CNS and peripheral nervous system (50). It likely plays important roles in glial-axon membrane interactions (51), therefore being an important target for neuronal regeneration or remyelination after CNS injury.

2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) is a membrane-anchored myelin enzyme that is exclusively and highly expressed by oligodendrocytes (52). In fact, it is the earliest known myelin-specific protein expressed by oligodendrocytes (53). Its exact physiological roles remain unknown, but studies involving the disruption of the *Cnp* gene in mice show that it is of great importance to myelin stability and neuronal health. Overexpression of CNPase inhibits MBP accumulation and myelin compaction (54), and is associated with age-dependent myelin degradation (55). Total absence of CNPase leads to age-dependent axonal degeneration but without myelin disruption (56, 57). Lipids constitute the vast majority of myelin: 70% to 85% of myelin's dry weight is composed of lipid. White matter is named as such because its lipid content gives it a whitish appearance. The most common lipid in myelin, galactocerebroside, is synthesized in oligodendrocytes and Schwann cells (58, 59). Krabbe disease, a rare and fatal lysosomal storage disease, arises from mutations of the gene that codes for galactocerebrosidase, *GALC* (60). Lacking this enzyme leads to a buildup of unmetabolized lipids and severe demyelination. Cholesterols are also important for the maintenance of cell membranes (61), and the brain is particularly rich in cholesterol: 25% of the body's total cholesterol content could be found within the brain (62), of which 80% is in myelin (63, 64). Perturbation of myelin synthesis in mice leads to ataxia and tremor phenotypes (64). Interruption of cholesterol or lipid synthesis pathways without altering myelin protein levels could subsequently lead to demyelination.

1.2.3 Demyelinating disorders

Disorders in myelin production or maintenance are called demyelinating disorders. The most prominent demyelinating disorder is MS, affecting close to 1 in 1,000 people in North America and Europe, and is the foremost disabling disease in young adults (65). MS is an inflammatory disorder in which immune cells attack and destroy myelin sheaths surrounding axons in the CNS, resulting in an impairment of neuronal transmissions and symptoms such as muscle weakness, optic neuritis, and cognitive impairment (66). Once damage to myelin has occurred, adult humans only have a very limited capacity to stimulate the remyelination in damaged tissue.

A particular subset of demyelinating disorders is known as leukodystrophies, a group of rare diseases caused by genetic abnormalities that lead to aberrant production or destruction of myelin. Leukodystrophies include Canavan disease (67), Pelizaeus-Merzbacher disease (42, 68) and adult-onset autosomal dominant leukodystrophy (ADLD) (69), each with different etiologies and ages of onset ranging from childhood to middle age. In all cases, treatments may improve quality of life, but there are currently no cures.

1.3 Laminopathies

Diseases from lamin mutations or misregulation are collectively known as laminopathies. *LMNA*-linked laminopathies include a wide variety of disorders in a wide variety of tissues and organ systems such as cardiomyopathy (70, 71), Emery-Dreifuss Muscular Dystrophy (72), Charcot-Marie-Tooth disease (73), Familial Partial Lipodystrophy (74), and the accelerated aging disorder Hutchinson-Gilford Progeria Syndrome (75). In contrast, B-type lamins are not associated with as many diseases as their A-type counterparts. *LMNB2* mutations have been associated with Acquired Partial Lipodystrophy (76), and the overexpression of *LMNB1* leads to ADLD (77).

1.3.1 Autosomal Dominant Leukodystrophy (ADLD)

ADLD (OMIM #169500) is a rare, progressively severe, and fatal demyelinating laminopathy with an average age of onset at the fourth or fifth decade of life. Patients with ADLD lose the protective myelin sheath surrounding their axons, resulting in muscle weakness, loss of autonomic function, paralysis, and eventually death (78). Distinct from MS in its severity, ADLD demyelination also occurs in a symmetrical fashion unlike the multifocal lesions commonly found in MS patients (79). We have further shown that lamin B1 overexpression in oligodendrocytes

leads to defects of lipid synthesis, eventually resulting in a severe age-dependent demyelinating phenotype characteristic of ADLD (80).



Figure 1.5: Tandem head-to-tail duplications of LMNB1 are associated with ADLD.

The established etiology of ADLD is that a genomic duplication of *LMNB1* leads to its overexpression (Figure 1.5). Molecular analyses of genomic duplications involving *LMNB1* attribute non-homologous end joining, microhomology-induced break replication, or other replication-based mechanisms as the cause of duplications (81-83).

Recently however, we have identified a few families with ADLD that do not have a *LMNB1* duplication (84). These families have large deletions upstream of the *LMNB1* locus instead, varying in size from 250 kb to 672 kb in size. The current hypothesis for the mechanism of *LMNB1* overexpression is that the deleted regions include a topological associating domain (TAD) boundary. TADs are sections of the genome enriched with frequently interacting DNA-DNA complexes that form within a region as a result of a chromosome's three-dimensional organization within the nucleus (85), identified through a process known as chromosome conformation capture (86). The exact mechanism of TAD formation remains poorly understood, but they often form within chromatin loops between lamina-associated domains at the nuclear periphery (87). TAD boundaries sequester one TAD from the next, so that the regulatory mechanisms such as enhancers in one TAD do not interact with genes present in a different TAD. Perturbations of TAD

boundaries could lead to aberrant chromatin interactions, and in the case of genomic deletions upstream of *LMNB1*, gene overexpression (88).

1.4 Public Health Significance

Laminopathies as a whole represent a wide variety of diseases that can affect nearly every tissue and organ system within the human body. One such laminopathy, the demyelinating disorder known as ADLD, has significant overlap with more prevalent demyelinating disorders such as MS (89). However, MS is a multifactorial disease resulting from the complex interactions of many genetic and environmental factors over long periods of time. Researching molecular pathways within rare Mendelian-inherited disorders involving one gene and one disease with high penetrance, such as ADLD, is more straightforward than disentangling complex, multifactorial diseases encompassing dozens of genes with varying contributions to the phenotype. Finding an effective treatment for white matter injuries would be of significant public health value. Impairment of oligodendrocyte maturation through hypoxia-ischemia and infection/inflammation during gestation can lead to cerebral white matter injury at birth, common in preterm neonates (90). In the aging brain, white matter is altered and degraded in dementias such as Alzheimer's disease (91, 92). Even though ADLD is very rare (fewer than 30 known affected families worldwide), interventions designed to halt disease progression may also be applied to MS, white matter injuries, and other demyelinating disorders. The role of LMNB1 also extends far beyond nuclear shape maintenance. For example, LMNB1 expression can regulate cell proliferation rates and senescence (93), with ramifications to cellular aging processes (94). In addition, lamin B1 degradation through autophagy has been proposed as a possible cellular defense mechanism

against carcinogenesis (95). Therefore, studying the interactions between lamins and their significance in white matter development and oligodendrocyte differentiation has far-ranging implications for public health.

1.5 Summary

The nuclear lamina functions to maintain nuclear shape and structure as well as regulate gene expression. Intermediate filaments known as lamins are organized in to two types: A-type and B-type. Each have diverse functions within various tissues and undergo distinct posttranslational modifications. Although their chemical makeup has been well-documented, the organization of the lamina at the nuclear periphery has been a mystery until now.

Glial cells known as oligodendrocytes are responsible for generating the protective myelin sheaths around axon fibers in the CNS. In ADLD, these cells are particularly affected, leaded to age-dependent demyelination. Overexpression of *LMNB1* in oligodendrocytes leads to ADLD-like symptoms in mice due to defects in lipid synthesis pathways. However, no studies have been done to explore the role of lamin B1 specifically in the proliferation, differentiation, or function of oligodendrocytes.

Mutations of lamin genes or changes in their expression often lead to diseases collectively known as laminopathies. Laminopathies involving the *LMNA* gene, which codes for both A-type lamins (A and C), are most often studied due to the wide variety of syndromes affecting a multitude of organ systems, including cardiomyopathy, muscular dystrophy, lipodystrophy, and progeria. In contrast, laminopathies involving B-type lamins are less numerous. One such laminopathy is ADLD, a severe and fatal demyelinating disorder caused by the overexpression of *LMNB1*, the

gene coding for lamin B1. Genomic duplications in 5q23 surrounding *LMNB1* are canonically accepted to be the mechanism for overexpression that causes ADLD. However, there is mounting evidence from my research that simply an extra copy of this gene is not the only factor needed to cause overexpression leading to ADLD.

My thesis work focuses on answering the following questions:

- 1.) What is the structural organization of A and B-type nuclear lamins at the nuclear periphery?
- 2.) What role does lamin B1 play in oligodendrocyte development and maturation?
- 3.) How do genomic duplications, deletions, or rearrangements involving LMNB1 lead to ADLD pathogenesis?

To address these questions, we have used super-resolution microscopy and biochemical analyses on human and murine cells in culture, generated transgenic mice with *Lmnb1* knocked out in oligodendrocytes, and compared copy number variants of families with ADLD against their clinical outcomes. This work will provide new paradigms for the nuclear organization and genomic regulation of *LMNB1*.

2.0 Hypotheses and Specific Aims

The overall aim of my dissertation research is to evaluate the function of lamin B1 in development and disease: its role within the nuclear envelope maintaining nucleus shape and stability, its function within oligodendrocytes, and finally tissue-specific genome regulation affecting its expression.

Aim 1: Decipher the structural and functional organization of the nuclear lamina.

Although the nuclear lamina is well-studied, how the lamin proteins are organized is still unknown. I hypothesize that the organization of individual lamin species will indicate their functions in maintaining envelope nuclear shape.

Methods: In collaboration with Dr. Yang Liu at the University of Pittsburgh and Dr. Kris Dahl at Carnegie Mellon University, we have used a combination of approaches including superresolution microscopy, conventional fluorescent microscopy, and various biochemical and biophysical techniques to determine the spatial organization of A-type and B-type lamins at the nuclear periphery of mouse and human cells. We will determine whether the organization of these individual lamin components provide insights into their distinctive functional roles.

Aim 2: Evaluate the role of lamin B1 in mouse oligodendrocyte development.

The function of lamin B1 specifically in oligodendrocytes has not been studied in detail. I hypothesize that the loss of *Lmnb1* in mouse oligodendrocytes will adversely affect their development and proliferation, leading to myelination defects in the CNS.

Methods: Using cre-lox recombination, I have generated mice with *Lmnb1* knocked out in mature oligodendrocytes. We will characterize the behavior of these mice, the expression of

myelin-associated genes at different time-points, and the properties of oligodendrocyte lineage cells using behavioral as well as biochemical and immunofluorescence analyses.

Aim 3: Study the role of genomic rearrangements involving *LMNB1* in the pathogenesis of ADLD.

Although *LMNB1* duplications are causative for ADLD, we have now identified families with ADLD without duplicated *LMNB1*, as well as families with *LMNB1* duplications but without ADLD. Different types of copy number variations surrounding *LMNB1* can have distinct consequences in terms of ADLD pathogenesis, so I hypothesize that there is a novel genomic regulatory pathway of *LMNB1* that provides a comprehensive mechanism predicting the various disease outcomes.

Methods: A custom microarray-based comparative genomic hybridization assay was used determine the copy number variations surrounding the *LMNB1* gene in 5q23.2. Duplication junctions were then identified, and insertion sites of the duplications mapped were back to the genome. The size and nature of these variations are matched with their clinical outcomes. We hope to narrow down a genomic region of interest for further study into the regulatory mechanisms of *LMNB1*. CRISPR knockout experiments could then be designed for future studies.

3.0 The Structural and Functional Organization of the Nuclear Lamina

3.1 Introduction

Studies utilizing optical microscopy and cryo-electron tomography (cryo-ET) to visualize the structural organization of lamins in mammalian cells revealed the presence of a meshwork structure, and indicated that each component formed separate but interacting meshworks (13, 96). However, no distinct spatial organization of the individual lamin subtypes across the nuclear envelope was reported at the time. It has been known that the depletion of specific lamin species, A-type or B-type, leads to disparate nuclear abnormalities *in vitro*. The removal of B-type lamins leads to an increase in the frequency of nuclei with blebs (13, 14), but the removal of A-type lamins leads to nuclear envelope ruptures and defective mechanotransduction (8, 97). While the effects of disease-causing *LMNA* mutations on nuclear shape and function has been well studied (98), the same cannot be said about *LMNB1* overexpression *in vitro*.

Previous research into the organization of the lamina at the nuclear periphery has been conducted with the use of electron microscopy (EM) (96, 99-101). While EM's resolution is a staggering 0.2 nm, a significant drawback is its inability to simultaneously image and discriminate different protein species. Limited protein differentiation could be achieved using immunogold particle labeling with variable-sized gold nanoparticles (96), but the size of the antibody-particle complexes (up to 30 nm) and their signal density compaed to fluorescent markers make differentiating closely-associated proteins like A-type and B-type lamins nearly impossible. On the other hand, conventional fluorescence microscopy can easily differentiate protein species simultaneously with the use of fluorophore-conjugated antibodies. However, its spatial resolution

is diffraction-limited to around 200 nm thanks to the properties of visible light wavelengths. This is much too large to resolve proteins that are potentially less than 15 nm apart.

I aim to determine whether the A and B type lamins exhibit any distinct spatial organization, and if so, whether this was reposonsible for the disparate consequences from depletion of the two lamin subtypes. To achive this aim, we used a combination of a super-resolution microscopy technique known as stochastic optical reconstruction microscopy (STORM), biochemical, and biophysical methods. STORM combines the ability to resolve past the diffraction limit while differentiating multiple proteins simultaneously. It offers the best spatial resolution of ~20 nm using relatively simple instruments, and the resolution of the final reconstructed image can be improved by an order of magnitude (102). STORM and biophysical studies were carried out in collaboration with Prof. Yang Lui's group at the University of Pittsburgh and Prof. Kris Dahl's group at the Carnegie Mellon University.

3.2 Materials and Methods

3.2.1 Cell Culture

Primary MEFs (Wild-type and *TRE-FLAG-LMNB1;ROSA26-rtTA* cells were isolated in lab from E13.5 embryos, *Lmnb1^{CS/CS}* cysteine-to-serine mutant (23), *Lmnb1^{-/-}* (9) and *Lmna^{-/-}* (103) cells were from the lab of S.G. Young), human fibroblasts, and HeLa cells were cultured in complete DMEM (high glucose DMEM (Corning) supplemented with 10% FBS (Fisher), 2mM L-glutamine (Millipore), and 1% Penicillin streptomycin (Hyclone)). Cells were incubated in a humidified chamber at 37°C and 5% CO₂. When cells reached 90-100% confluence, they were

trypsinized, diluted, and re-plated using fresh media. For calyculin A and blebbistatin treatments, HeLa cells grown on coverslips were treated with 0.1 and 1 nM calyculin A (Sigma #508226) for 30 minutes, or 10, 50, and 100 μ M blebbistatin (Sigma #203389) for 2 hours at 37°C and 5% CO₂. Cells were washed and fixed in 4% paraformaldehyde solution in PBS immediately after treatment. Lamin B1 overexpression was induced in *TRE-FLAG-LMNB1;ROSA26-rtTA* transgenic MEFs by supplementing growth medium with 2 μ g/mL doxycycline hyclate (Sigma).

3.2.2 STORM imaging and analysis

Cells for STORM imaging were prepared as previously described (104). Rabbit anti-lamin B1 (Abcam #ab16048) were diluted 1:600 and/or mouse anti-lamin A/C (Cell Signaling #4777) were diluted 1:300 in PBS + 3% BSA, and incubated on the cells overnight at 4° C. The next morning, cells were washed with PBS three times for 5 minutes each at room temperature. Secondary antibodies were diluted in PBS + 3% BSA and incubated with the cells for 2 hours at room temperature in the dark. Unconjugated secondary antibodies (Jackson ImmunoResearch #711-005-152 and #715-005-150) were conjugated with AlexaFluor (AF) 647 (Thermo Fisher Scientific, #A20006) in the lab of Dr. Yang Liu, University of Pittsburgh. AF647-conjugated secondary antibodies were used for single-color STORM imaging. For two-color STORM imaging based on dye pairs, secondary antibodies labeled with activator-reporter dye pairs (AF405-AF647, Cy2-AF647) were used. AF405-AF647 conjugated donkey anti-mouse secondary antibody was used to label lamin A/C, and Cy2-AF647 conjugated donkey anti-rabbit secondary antibody was used to label lamin B1. For the dye-switch experiment, AF405-AF647 conjugated donkey anti rabbit secondary antibody was used to label lamin B1, and Cy2-AF647 conjugated donkey anti mouse secondary antibody was used to label lamin A/C. Immediately before imaging, the buffer was switched to STORM imaging buffer (10% w/v glucose (Sigma-Aldrich), 0.56 mg/mL glucose oxidase (Sigma-Aldrich), and 0.17 mg/mL catalase (Sigma-Aldrich)). For single color imaging, 0.14M β-mercaptoethanol (Sigma-Aldrich) was used, and for two-color imaging, 0.1M mercaptoethylamine (MEA, Sigma-Aldrich) was used.

Single-color STORM imaging was performed on a custom-built system using an Olympus IX71 inverted microscope frame with a 60X oil objective. Fluorescent beads (0.1 µm diameter, Fisher Scientific #F8803, excited using 488 nm laser) are used as fiduciary markers on the coverslip to correct for 3-D system drift every 200 frames. Two-color STORM images using dye pairs were acquired on a commercial imaging system (N-STORM, Nikon Instruments). The samples were periodically activated with a sequence of 405nm, 488nm laser pulses and then imaged with the 647nm laser. In each switching cycle, the activation laser was turned on for 1 frame, followed by 3 frames of illumination with the red imaging laser. A total of 40,000 frames, including 10,000 activation frames and 30,000 imaging frames for each channel, were acquired at an exposure time of 20 milliseconds per frame. Imaging frames immediately following an activation pulse were recognized as controlled activation events and a color was assigned accordingly. A crosstalk subtraction algorithm was used to subtract the non-specific activation signal (105).

Nuclei were imaged at the equator for measuring lamina thickness and relative localization. For lamina surface imaging, the bottom surfaces of nuclei were imaged in order to minimize curvature artifacts. The reconstruction of a super-resolution image and Gaussian clustering were performed using a custom program written in Matlab 2015 (MathWorks), as previously described (106). The degree of co-localization was calculated using Clus-DoC algorithm (107) and defined as the degree of co-localization in lamin A/C and lamin B1 with respect to the combined lamin A/C and lamin B1. For measuring lamina thickness, the nuclear periphery of the super-resolution image was automatically divided into numerous small segments at a length of ~50 nm. Intensity peaks were measured along the steepest gradient perpendicular to the nuclear envelope, and averaged. The intensity profile was plotted as distance (x axis) versus normalized lamin intensity (y axis), and thicknesses were defined as the full width at half maximum (FWHM).

3.2.3 Isolation of nuclei

Low-passage cells were seeded in 100-mm tissue culture dishes (Thermo BioLite) and grown to 90% confluence. Nuclei were isolated as previously described (108), with modifications. The cells were washed once with 3 mL ice-cold PBS, then scraped down with 1 mL cold PBS into 1.5 mL microcentrifuge tubes. Samples were centrifuged at 10,000xg for 15 seconds at 4°C, and the supernatants were discarded. Cell pellets were resuspended in 1 mL cold PBS+IGEPAL (1x PBS + 0.1% IGEPAL CA-630 (Sigma) + protease inhibitors), triturated 5x on ice with a 1000 μ L pipet tip, and 100 μ L transferred into new microcentrifuge tubes as the whole cell fraction. Remaining volumes were centrifuged at 10,000xg for 15 seconds at 4°C. Supernatants were transferred to new microcentrifuge tubes labeled "cytosol." Pellets were resuspended in 1 mL PBS+IGEPAL, then centrifuged at 10,000xg for 15 seconds at 4°C, and the supernatants were discarded. Remaining pellets were used for sequential protein extractions. Cytosol samples were centrifuged at 10,000xg for 1 minute at 4°C, then 300 μ L of the supernatant was transferred to new microcentrifuge tubes as the clean cytosolic fraction.

3.2.4 Sequential extraction of nuclear envelope proteins

Subfractionation of nuclear proteins was performed as previously described (109). Nuclear pellets were resuspended in 300 µL nuclear isolation buffer (10 mM HEPES pH 7.4, 2 mM MgCl₂, 25 mM KCl, 250 mM sucrose, 1 mM DTT, protease inhibitors). 50 µL of each sample were transferred to new microcentrifuge tubes as the "whole nuclei" fraction. Samples were sonicated on ice with two 5 second pulses at 10 µm amplitude, then centrifuged at 20,000xg for 5 minutes at 4°C. The supernatants were transferred to new microcentrifuge tubes as the "Sonication" fraction. Pellets were resuspended in 250 µL nuclear extraction buffer (20 mM HEPES pH 7.4, 1 M NaCl, protease inhibitors), and incubated for 20 minutes at room temperature with end-over-end rotation. After incubation, samples were centrifuge tubes as the "1 M NaCl" fraction. Extractions were repeated on the pellets using 250 µL nuclear extraction buffer with 2% v/v Triton X-100, 4 M urea, and then 8 M urea in sequential incubations. All protein extracts were stored at -80°C until ready for immunoblotting.

3.2.5 RNAi knockdown of lamin genes

6-well plates were seeded with 4x10⁵ HeLa cells/well in DMEM without serum or antibiotics and transfected for 24 hours with 50 nM siRNA (Scrambled: ThermoFisher #4390843, *LMNB1*: ThermoFisher #S8224 and #S8225, *LMNA*: ThermoFisher #S8221) using RNAimax transfection reagent (ThermoFisher) diluted in OptiMEM (Gibco). The next day, cells were refed with complete DMEM and incubated for another 24 hours, after which proteins were extracted using T-PER with protease inhibitors (Thermo), or immunocytochemistry was performed.
3.2.6 Immunofluorescence staining and analysis

Cells were seeded on glass coverslips and incubated at 37° C and 5% CO₂ until they were 70-90% confluent. Cells were washed with DPBS (Sigma), then fixed with 4% formaldehyde (Ted Pella) in PBS for 15 minutes at room temperature. After three washes of 5 minutes each with PBS, blocking and permeabilization was performed simultaneously with PBS + 5% normal donkey serum (Jackson ImmunoResearch) + 0.3% Triton X-100 (Sigma) for 1 hour at room temperature. Primary antibodies were diluted in PBS + 1% BSA (Sigma) + 0.3% Triton X-100 and incubated with the cells overnight at 4°C. Primary antibodies and dilutions are listed in Appendix A. The next morning, primary antibody dilutions were discarded, and cells were washed with PBS three times for 5 minutes each at room temperature. FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch #711-095-152) and Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch #715-165-150) secondary antibodies were each diluted 1:350 in PBS + 1% BSA, and incubated with the cells for 1 hour at room temperature in the dark. Cells were washed three times for 5 minutes each in PBS, then mounted onto glass microscope slides using Vectashield antifade mounting medium with DAPI (Vector Laboratories).

Wide-field microscopy images were acquired of immunofluorescently-labeled nuclei on a Leica DM5000B upright microscope with a 40x NA 0.75 objective, a 63x NA 1.40 oil immersion objective, and a Leica DFC310 FX digital camera. Confocal images were acquired on an Olympus FV1000 inverted microscope using a 60x NA 1.40 low chromatic aberration oil immersion objective and integrated digital camera. ImageJ (FIJI) was used to measure nuclear periphery/center (P/C) fluorescence intensity ratios and fluorescence intensity profile plots from confocal images. P/C ratios were calculated by measuring the mean gray value of the nuclear periphery and dividing it by the mean gray value of the nuclear center in red and green channels

separately. Average P/C ratios from at least 15 nuclei per genotype (WT versus *Lmnb1^{CS/CS}* MEFs) were plotted with standard errors. Fluorescence intensity profile plots were generated by drawing a line across the midline of a nucleus and plotting gray value versus distance. Nuclear bleb ratios were calculated by counting the number of nuclei with one or more blebs in wide-field images, and dividing it by the total number of nuclei imaged. Blebs were considered devoid of a lamin if its intensity was less than 50% of the intensity throughout the nuclear body after background fluorescence subtraction. At least 150 nuclei per cell type and treatment were counted in 15 to 20 random, non-overlapping fields. Partially-visible nuclei at the field edges were ignored.

3.2.7 Immuno-TEM and analysis of mouse embryonic fibroblasts

MEFs were grown to confluency in 24 well tissue culture plates, washed once with 37°C PBS then fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in PBS for 1 hr at RT. Cells were washed 3 x with PBS then permeabilized for 30 min at RT with 0.25% Triton X-100 in PBS. Cells were washed 3x in PBS then blocked in 5% normal goat serum in PBS for 45 min. Cells were washed 2x for 15 min in 0.5% BSA, 0.1% Tween-20 in PBS (BT-PBS) then incubated with individual anti-lamin primary antibodies (1:25 dilution in BT-PBS) 18 hrs while shaking at 4°C. Cells were washed 5 x in BT-PBS 10 min each, then 6 nm gold-conjugated antibodies (goat antirabbit or goat anti-mouse, (Jackson ImmunoResearch, West Grove, PA) diluted 1:10 in BT-PBS) were incubated for 18 hr at 4°C while shaking. Following 3x 15 min washes with BT-PBS, and additional 3 x 15 min washes in PBS, labeled cells were fixed with 2.5% glutaraldehyde in PBS for 1 hr. Cells were then washed 3 x in PBS, post-fixed in 0.5% osmium tetroxide in dd-H₂O for 30 min. Following 3 washes in PBS, cells were then dehydrated through a 30-100% ethanol series then several changes of Polybed 812 embedding resin (Polysciences, Warrington, PA). Cultures

were embedded in by inverting Polybed 812-filled BEEM capsules on top of the cells. Blocks were cured overnight at 37°C, then cured for two days at 65°C. Monolayers were pulled off the TC plate and ultrathin en face sections (60 nm) of the cells were obtained on a Riechart Ultracut E microtome, post-stained in 4% uranyl acetate for 10 min and 1% lead citrate for 7 min. Sections were viewed on a JEOL JEM 1011 transmission electron microscope (JEOL, Peabody MA) at 80 KV. Images were taken using a side-mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

To calculate the distance between lamin A/C to chromatin boundary, we first defined the positions for nanogold particles and chromatin boundary. To define the position for the nanogold particles, we processed the images as follows: (1) invert the grayscale value of the EM image; (2) perform image smoothing using difference of Gaussian filter; (3) find the local maxima as the position of nanogolds; (4) the central positions of the identified nanogolds along the nuclear membrane is retrieved by finding a curve with minimum deviation to the positions of nanogold by adapting a 2nd order polynomial function; (5) fit the accumulated number of the segmented nanogold particles along the central trajectory of nanogolds with a Gaussian function and the peak position for chromatin boundary, we first remove the segmented nanogold particles as described above from the EM image, plot the average intensity profile of the remaining EM image along the central trajectory identified above and the peak position of the intensity profile is used as the position for chromatin boundary. The distance between the positions of lamin A/C and chromatin boundary, and lamin B1 and chromatin boundary was then calculated.

3.2.8 Micropipette aspiration

HeLa cells were seeded into glass-bottomed dishes in complete DMEM and cotransfected with DsRed-LMNA and GFP-LMNB1 plasmids using Lipofectamine3000 (ThermoFisher), according to the manufacturer's protocol for double transfections. Cells were imaged and manipulated 48 hours post transfection, and 0.17 mg/mL of Hoescht 33342 (ThermoFisher) was added to the media to visualize DNA. Live cell imaging in micropipette aspiration experiments was performed on a Leica DMI6000 inverted microscope with a 40x NA 1.25 oil immersion objective, and a Leica DFC350 FX digital camera. Micropipettes were pulled from 1 mm glass capillary tubes using a PMP102 Micropipette puller (Microdata), and the micropipette used for the study was 5.8 µm in diameter. Narishige pipette holders and micromanipulators were used to approach the cells of interest, and nuclei were aspirated directly from intact cells. Aspiration pressure was applied via a syringe to obtain aspirations of different lengths. Analysis of intensities and lengths was performed in ImageJ. The intensity at the aspiration tip, which is the curved section of the nuclear lamina farthest down the pipette, was normalized to the intensity of the nonaspirated region of the lamina. Note that the intensity of the DsRed-lamin A/C was 150-200% brighter than that of GFP-lamin B1.

3.2.9 Immunoblotting

Protein concentrations were measured using the Pierce BCA protein assay kit (ThermoFisher). 70 μ g of protein was mixed with 4x Laemmli sample buffer (Bio-Rad) and boiled for 10 minutes at 95°C. For sequential nuclear extraction samples, 18 μ L of each fraction was mixed with 6 uL of 4x Laemmli sample buffer, and boiled for 10 minutes at 95°C. Protein samples

were then separated using SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad), then blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR Biosciences). Primary antibodies were diluted in at the following concentrations: Membranes were hybridized with primary antibodies diluted in Odyssey blocking buffer + 0.1% Tween 20 (Fisher) overnight at 4°C with gentle shaking. Primary antibodies used and their dilutions are listed in Appendix A. The next morning, membranes were washed three times for 10 minutes each with TBS-T (TBS + 0.1% Tween 20), and incubated with goat anti-mouse 680LT (LI-COR #926-68020) and donkey anti-rabbit 800CW (LI-COR #926-32213) secondary antibodies diluted 1:10000 in Odyssey blocking buffer + 0.1% Tween-20 for 1 hour at room temperature in the dark. After three 10minute washes in TBS-T, membranes were scanned, imaged, and quantified using the LI-COR Odyssey CLx infrared scanner and Image Studio software (LI-COR Biosciences).

3.2.10 Statistical analysis

Two-sided *t*-tests were used to calculate statistical significance between two groups. For micropipette aspiration comparisons, two-way ANOVAs were used followed by Sidak's multiple comparisons test. For sequential protein extractions, lamin signal percentages were arcsine transformed prior to statistical analysis. Chi-squared tests were used to assess statistical significance of blebbing ratios. Bleb versus nuclear lamin intensity differences were compared using Wilcoxon matched-pairs signed rank test. A p-value ≤ 0.05 was considered statistically significant. The following convention for representing p values was followed: *p ≤ 0.05 , **p ≤ 0.01 , ***p ≤ 0.001 . All error bars represent standard error unless otherwise specified. Data were graphed and analyzed in Microsoft Excel 2016 and GraphPad Prism 7.

3.3 Results

3.3.1 Differential localization and membrane association of lamin B1 versus lamin A/C

To visualize the spatial relationship between lamin A/C and B1, we used two-color STORM imaging carried out by Prof. Yang Liu's group at the University of Pittsburgh. Cells were immuno-stained with primary antibodies against nuclear lamina proteins lamin A/C and lamin B1, and then with secondary antibodies conjugated with activator-reporter dye pair (110) (Cy2-AlexaFluor 647 and AlexaFluor 405-AlexaFluor 647). The use of the same reporter dye (AlexaFluor 647) eliminates chromatic aberration, which is crucial for the precise localization of the two types of lamins. We specifically focused on the equatorial plane of the cell as the superior resolution in the x-y axes would allow us to better appreciate differences in spatial localization between the lamin A/C and B1 species across nuclear periphery. We discovered that lamin B1 preferentially localizes closer to the inner nuclear membrane (INM) whereas lamin A/C is localized closer toward the nucleoplasm; there is also significant spatial overlap between the Atype and B-type lamina meshworks. This pattern was consistent across different cell types and species including primary mouse embryonic fibroblasts (MEFs) (Figure 3.1A and B), HeLa cells (Figure 3.1C and D), and primary human fibroblasts (Figure 3.1E and F). Lamin B1 was localized closer to the INM compared to lamin A/C along the entirety of the nuclear periphery.

To independently test association of lamin B1 with the INM, we subjected isolated MEF nuclei to extraction buffers of increasing stringency that sequentially isolate proteins from the lipid-bound fractions. We observed that lamin B1 requires more stringent conditions for its release into solution. In the two least stringent conditions, 80% of the total lamin A/C is extracted,



Figure 3.1: Spatially distinct localization of lamin B1 and lamin A/C.

STORM images of immunofluorescently-labeled lamin B1 (green) and lamin A/C (red) nuclear proteins in (A) MEF, (C) HeLa, and (E) human fibroblast nuclei at their equatorial planes. Scale bar: $2 \mu m$. Rectangles denote inset zoomed areas. Inset scale bars: 500 nm and 100 nm. (B), (D), and (F) Fluorescence intensity profile plots across the nuclear envelopes in STORM images. X-axis: distance (nm). Zero distance denotes center of nuclear lamina Y-axis: intensity (arbitrary units). Inset: Box-and-whisker plot of separation (nm) between lamin A/C and B1 fluorescent intensity peaks. n = 5 nuclei. Storm imaging carried out by Dr. Jianquan Xu and Prof. Yang Liu, University of Pittsburgh.

compared to only 40% of lamin B1 in MEFs (Figure 3.2A and C). Since A-type and B-type lamin proteins have similar percentages of hydrophobic amino acids (lamin A: 32%, lamin C: 33%, lamin B1: 34%), differences in extraction profiles are most likely due to differences in membrane association and not hydrophobicity. We replicated these results in HeLa cells (Figure 3.2B and D), and they are consistent with our STORM imaging data that lamin B1 is more closely associated with the INM as compared to lamin A/C. For further confirmation, we carried out electron microscopy with immunogold labeling of lamin A/C and B1 proteins (Figure 3.3). These experiments clearly demonstrated a more peripheral localization for lamin B1.



Figure 3.2: Lamin B1 is more tightly associated with the inner nuclear membrane.

Representative western blots of sequential nuclear protein extractions in (A) MEF and (B) HeLa cells. (C) Quantification of fractional amount of nuclear lamin proteins solubilized in increasingly stringent sequential extractions in WT MEFs and (D) HeLa cells. Graphs represent fraction of total lamin B1 and lamin A/C signal in each extraction from three independent experiments. Bars, mean \pm S.E.M.; *p \leq 0.05, **p \leq 0.01; unpaired two-tailed t-test.



Figure 3.3: Electron microscopy confirms concentric lamin organization.

Representative electron microscopy images of MEF cells labeled with 6 nm gold nanoparticles conjugated to antibodies against (A) lamin A/C and (B) lamin B1. Images were analyzed and profiles generated as described in methods section. The representative normalized profile of chromatin boundary vs. (C) lamin A/C and (D) lamin B1 are shown. Arrows indicate distance between chromatin boundary and lamin profiles. TEM processing and imaging courtesy of Donna Stolz, Center for Biologic Imaging.

3.3.2 The C-terminal farnesyl group is necessary for lamin B1 localization

Given that lamin B1 retains its farnesyl group, we sought to test whether this moiety might be responsible for its association with the INM and examined MEFs derived from mice homozygous for a cysteine-to-serine mutation ($Lmnb1^{CS/CS}$) in the CAAX domain which inhibits



Figure 3.4: The C-terminal farnesyl group determines the spatial organization of lamin B1.

(A) STORM image at equatorial plane of an immunofluorescently-labeled $Lmnb1^{CS/CS}$ MEF nucleus. Scale bar: 2 µm. Rectangles denote inset zoomed areas. Inset scale bars: 500 nm and 100 nm. Lamin B1 (green), lamin A/C (red). (B) Fluorescence intensity profile plots across the STORM imaged $Lmnb1^{CS/CS}$ MEF nuclear envelope. X-axis: distance (nm). Y-axis: intensity (arbitrary units). Inset: Box-and-whisker plot of separation (nm) between lamin A/C and B1 fluorescent intensity peaks from 5 nuclei. (C) Comparison of lamin B1 and lamin A/C intensity peaks separation in WT vs $Lmnb1^{CS/CS}$ MEFs. n = 5 nuclei. (D) Confocal immunofluorescence images of representative WT and $Lmnb1^{CS/CS}$ MEF nuclei taken at the equatorial plane. Lamin B1 (green), lamin A/C (red). Scale bar: 10 µm. Dotted lines depict measurement lengths of fluorescent intensity plots shown in Fig. 3.4E. (E) Fluorescence intensity profile plots across representative WT and $Lmnb1^{CS/CS}$ nuclei. The sharp peaks at each end of the lamin A/C and WT lamin B1 plots represent bright peripheral staining. X-axis: measurement distance (µm). Y-axis: fluorescence intensity (arbitrary units). (F) Comparison of sequential lamin B1 extractions in WT vs. $Lmnb1^{CS/CS}$ cells. Graph represents fraction of total lamin B1 signal in each extraction from three independent experiments. Bars, mean \pm S.E.M; **p≤0.01, ***p≤0.001; unpaired two-tailed t-test. farnesylation of lamin B1 (23). STORM images of *Lmnb1^{CS/CS}* MEFs showed that lamin B1 was no longer localized closer to the INM (Figure 3.4A) and the relative intensity profiles revealed a lamin B1 signal completely overlapping with that of lamin A/C (Figure 3.4B and C).



Figure 3.5: Lack of lamin B1 farnesylation does not affect lamin A/C.

(A) Diagram of nuclear periphery and nuclear center in a confocal image of a representative WT MEF nucleus at the equatorial plane. (B) Graph of normalized Periphery/Center (P/C) intensity ratio. The P/C ratio of lamin B1 is significantly reduced in $Lmnb1^{CS/CS}$ MEFs, whereas lamin A/C is unchanged compared to WT. n = 30 WT, 15 $Lmnb1^{CS/CS}$. (C) Representative western blot of sequential nuclear protein extractions in $Lmnb1^{CS/CS}$ MEFs. (D) Comparison of sequential lamin A/C extractions in WT vs. $Lmnb1^{CS/CS}$ cells. Graph represents fraction of total lamin A/C signal in each extraction from three independent experiments. Bars, mean \pm S.E.M.

Confocal microscopy of *Lmnb1*^{CS/CS} nuclei at their equatorial planes demonstrated an abnormal distribution of lamin B1 throughout the nucleus (Figure 3.4D), including abnormally high lamin B1 levels in the nucleoplasm, whereas lamin A/C appears to be unaffected (Figure 3.4E). Quantifying the ratio of fluorescence intensity at the nuclear periphery "P" to the intensity at the nuclear center "C" (Figure 3.5A), the P/C ratio of lamin B1 is significantly smaller in *Lmnb1*^{CS/CS} MEFs compared to WT MEFs (Figure 3.5B), whereas lamin A/C ratios remains unchanged. We measured extraction of lamin proteins from nuclei isolated from *Lmnb1*^{CS/CS}

MEFs, finding that a striking 89.4 \pm 3.1% of the total lamin B1 was extracted in the least stringent fraction (Figure 3.4F and 3.5C), compared to 29.6 \pm 8.7% in WT cells and is consistent with previous results using farnesyltransferase inhibitors (111). Lamin A/C extraction profiles remained unchanged across WT and *Lmnb1*^{CS/CS} cells (Figure 3.5D). These data confirmed the STORM imaging results, indicating that the farnesyl group of lamin B1 is required for its proper localization to the INM.

3.3.3 Lamin B1 localization at the nuclear envelope is curvature dependent

Wide-field fluorescence images of WT MEF nuclei revealed a differential localization of lamin B1 and lamin A/C at the nuclear rim (Figure 3.6A): in more ellipsoidal nuclei, lamin B1 intensity was reduced at the poles. We quantified local radius of curvature, R, as a function of normalized lamin intensity (Figure 3.7A for methodology) across WT MEF nuclei. The intensity profiles of lamin staining with respect to R showed no correlation with lamin A/C, but an exceptionally strong correlation with lamin B1 (Figure 3.6B). Regions of lower R had reduced lamin B1 intensity and an extrapolation of the fit line to zero lamin B1 intensity suggests that curved structures with a radius of less than 1.8 µm would be devoid of lamin B1.

Alternatively, plotting of lamin intensity versus bending energy of the nuclear envelope can be approximated by the equation for a two-dimensional network: $E_{bend} = (\frac{1}{2}\kappa \frac{1}{R^2})$ (112, 113). Since the bending modulus κ is a constant material property, we plot the effective bending energy as a function of $1/R^2$ (Figure 3.7B). Lamin A/C localization is independent of lamina bending energy. In contrast, lamin B1 intensity is an extremely strong function of bending energy. An



Figure 3.6: Lamin B1 meshwork is curvature and strain-responsive.

(A) Wide-field fluorescent microscopy images of WT MEF nuclei displaying differential localization of lamin B1 and lamin A/C across the nucleus. Dashed lines represent major axes. The vertices at the ends of the major axis are defined as the nuclear poles. Arrowheads point to areas of decreased lamin B1 intensity. Scale bar: 10 μ m. (B) Plot of normalized lamin intensity versus radius of curvature R for 30 points across 17 nuclei, with logarithmic trendlines. At the x-intercept, R = 1.8 μ m. (C) MEFs were doubly transfected with *GFP-LMNB1* and *DsRed-LMNA* plasmids, then aspirated with a micropipette. Normalized aspiration length, L/D, is defined as the aspirated projection length, L, divided by the diameter of the micropipette, D. Arrows point to aspiration tip and base. Scale bar: 10 μ m. (D) Graph of lamin intensities at the aspiration tip versus L/D. n = 5. Lamin intensities at aspiration tip were normalized to its intensity throughout the rest of the nucleus. Bars, mean ± S.E.M; *p≤0.05, **p≤0.01, ***p≤0.001; two-way ANOVA with Sidak's multiple comparisons test. Curvature-intensity plots and micropipette aspiration experiments were carried out by Travis Armiger and Prof. Kris Dahl, Carnegie Mellon University.

elongated nucleus forms a tight curve (i.e. high bending energy) at polar ends, as a result, these regions are more likely to be devoid of lamin B1. Consistent with our epifluorescence data, STORM images of elongated MEF nuclei also show the characteristic loss of lamin B1 at the polar ends (Figure 3.7C). To confirm the strain-dependent localization of the A- and B-type lamins, we

used micropipette aspiration to induce and increase strain and visualize lamin intensity at the tip in the pipette, which is the region of highest strain (114) (Figure 3.6C).

Micropipette aspiration experiments were carried out by Prof. Kris Dahl's group at Carnegie Mellon University. At small aspiration lengths, normalized GFP-lamin B1 intensity is higher than normalized DsRed-lamin A intensity at the tip, which suggests that the stiff lamin A is resistant to deformation, as expected, compared to lamin B1. With increasing aspiration length, the normalized GFP-lamin B1 intensity significantly decreases at the tip, while normalized DsRedlamin A intensity remains relatively unchanged (Figure 3.6D). These findings are consistent with our imaging results and indicate that the network deformation of lamin B1 is influenced by strain at the pipette tip.



Figure 3.7: Relationship between lamin intensity and bending energy.

(A) Schematic representation of the relationship between curvature and its corresponding radius, R. A smaller R defines a tighter curve, and R approaches infinity as the curve approaches a straight line. (B) Plot of normalized lamin intensity versus effective bending energy for 30 points across 17 nuclei. Effective bending energy is proportional to $1/R^2$, where R is the radius of curvature. (C) Representative STORM images of a MEF at the nuclear equator showing typical loss of lamin B1 at the polar ends of elongated nuclei. Scale bar: 2 μ m.

3.3.4 Bleb architecture is dependent upon lamin levels and localization

One physiological consequence of curvature-dependent localization of the A- and B-type lamins is evident in the structure of nuclear blebs, which are characteristic outward protrusions of the nuclear lamina. Blebs were initially reported as a consequence of nuclear lamin perturbations, most pronounced in lamin B1-null and HGPS cells (13, 103, 115). However, we observed that these blebs were also present in a fraction of wild type MEFs (19.1 \pm 2.9%; Figure 3.8A and B) and untreated HeLa cells (12.2 \pm 1.7%; Figure 3.9C). Plotting the ratio of bleb lamin fluorescence intensity to nuclear fluorescence intensity reveals that most blebs were devoid of lamin B1 (Figure 3.8C). All nuclear blebs contained lamin A/C, while only a subset also contained lamin B1 (16.4 \pm 2.5% in MEFs, 30.4 \pm 3.0% in HeLa cells; Figure 3.8D). No blebs contained lamin B1 without lamin A/C also present. In the blebs that did contain lamin B1, the normalized fluorescence intensity of lamin B1 within the bleb was significantly lower than that of lamin A/C (Figure 3.8E), also suggesting that lamin B1 is depleted from blebs.

To determine whether altering levels of the different lamin subtypes had any effect on bleb frequency, we then examined bleb formation in WT, *Lmnb1*^{-/-}, and *Lmna*^{-/-} MEFs (Figure 3.9A and B). Interestingly, we observed that while *Lmna*^{-/-} MEFs were highly elongated and displayed ragged gaps at the polar ends, no blebs were present. In contrast, we observed a marked increase of bleb frequency in *Lmnb1*^{-/-} MEFs. *Lmnb1*^{CS/CS} MEFs also had more frequent blebbing nuclei, but not to the degree seen in *Lmnb1*^{-/-} cells (Figure 3.8B). In agreement with the lamin-null MEF data, siRNA-induced reduction of lamin B1 increased bleb frequency while the reduction of lamin A decreased bleb frequency (Figure 3.9C). Therefore, a possible role of the outer lamin B1 meshwork may be to prevent the A-type lamins underneath from expanding outward and forming nuclear envelope protrusions.



Figure 3.8: Lamin structure determines its roles in maintaining nuclear shape.

(A) Confocal images of a WT MEF nucleus at the equatorial plane showing a typical nuclear envelope protrusion referred to as a bleb (arrow). Dashed line represents major axis. (B) Bleb frequency quantification among WT (n=178), *Lmna^{-/-}* (n=178), Lamin B1-overexpressing (n=182), *Lmnb1^{CS/CS}* (n=201), and *Lmnb1^{-/-}* (n=201) MEF nuclei. (C) Histogram of bleb lamin intensity relative to nuclear body intensity in WT MEFs (n=214). Median ratios: 1.34 (LA/C), 0.10 (LB1). (D) Frequencies of blebs that contain LA/C only vs. both LA/C + LB1 in primary MEFs (n=225) and HeLa cells (n=230). (E) Box-and-whisker plot of lamin fluorescence intensity ratios among blebs that contain both lamin A/C and lamin B1 in WT MEFs (n=24). (F) Nuclear bleb frequencies in HeLa cells treated with 0.1 nM (n=288) and 1 nM Cal-A (n=324), compared to DMSO-treated control (n=220). (G) Radius measurements of blebs containing only lamin A/C (n=158) vs. both A/C and B1 (n=31). (H) Radius measurements of blebs in WT (n=189) vs. *Lmnb1^{-/-}* (n=87) MEFs. (I) Percentages of polar-localized blebs in WT (n=206) vs. *Lmnb1^{-/-}* (n=87) MEFs. Bars, mean ± S.E.; *p≤0.05, **p≤0.01, ***p≤0.001; Chi-squared tests were used for B, F, and I; Wilcoxon matched-pairs signed rank test for E, and unpaired two-tailed t-test with Welch's correction for G and H.

We hypothesized that rather than solely arising as a consequence of nuclear lamin perturbations, bleb formation may be a mechanism to redistribute tension on the lamina due to cytoskeletal forces exerted on the nucleus. To test this, HeLa cells were treated with 0.1 nM and 1 nM Calyculin A (Cal-A), a potent PP1 and PP2A-C phosphatase inhibitor that induces contraction of the actin-myosin cytoskeleton (116). Intensifying intracellular stress to promote rapid nuclear compression may lead to an increase of bleb frequencies. Cal-A treatment indeed resulted in increased bleb frequencies over control cells in a dose-responsive manner (Figure 3.8F). The short treatment period (30 min) ensures that the differences in bleb frequencies are not due to nuclear reorganization induced by cell division. To confirm that the increased blebbing frequency was due to increased actin-myosin contractility and not due to due to off-target phosphorylation caused by Cal-A treatment, we used the cell-permeable myosin II inhibitor, blebbistatin, to reduce intracellular pressure by suppressing cell contraction. Blebbistatin treatment resulted in a dosedependent decrease of blebbing nuclei (Figure 3.9D). Notably, it eliminated Cal-A's ability to induce blebbing, indicating that blebs likely arose due to increased intracellular pressure and not from non-specific effects of calyculin treatment.

The architecture of the blebs allows us to test predictions that arise out of our model of the nuclear lamina, where the lamin B1 network is located towards the outside of lamin A/C, and the location of lamin B1 is curvature dependent. Based on our findings, we expect that that lamin B1 is likely to be excluded from tightly curved regions of the nuclear envelope. Consistent with this prediction, we found that blebs containing lamin B1 are larger on average than lamin A/C-only blebs (Figure 3.8G). In addition, blebs that form in *Lmnb1*^{-/-} MEFs are significantly larger than those in WT cells (Figure 3.8H). Further proof of the functional consequences of our model is seen in the location of blebs. If a role for lamin B1 is to stabilize the outward protrusion of the lamin

A/C network, we would expect to see a higher frequency of blebs in regions depleted of lamin B1. In agreement with our model, we observe that most blebs form at the major axis poles, where the radius of curvature is low resulting in a consequent depletion of lamin B1. However, in $Lmnb1^{-/-}$



Figure 3.9: Nuclear bleb rates are altered with lamin perturbations.

(A) Epifluorescence microscopy images of WT, $Lmnb1^{-/-}$, and $Lmna^{-/-}$ MEF nuclei stained against lamin B1 (green), lamin A/C (red), and DNA (blue). Arrows point to a nuclear envelope protrusion known as a bleb. Arrowheads point to areas of nuclear envelope ruptures with herniating chromatin. Scale bar: 10 µm. (B) Western blot of lamin proteins in $Lmna^{-/-}$, $Lmnb1^{-/-}$, and WT MEFs. (C) Quantification of nuclear bleb ratios among untreated control (n=386), siRNA scrambled (n=117), siRNA LMNA (n=206), and siRNA LMNB1 (n=159) treated HeLa cells. (D) Blebbistatin treatments of HeLa cells result in a dose-dependent reduction of blebbing nuclei frequencies compared to control. 1 nM Cal-A co-treated with 50 µM blebbistatin results in the elimination of Cal-A-induced bleb formation. n = 250 -400 nuclei per group. (E) Western blot of WT vs. *TRE-FLAG-LMNB1;Rosa-rtTA* (TRE) transgenic MEFs that overexpress exogenous human FLAG-lamin B1 after the addition of 2 µg/mL doxycycline (+Dox) in vitro. Exogenous FLAG-lamin B1 (top band), endogenous lamin B1 (bottom band). M = protein size marker, with relevant band sizes labeled (kDa). (F) Nuclear bleb frequencies in WT vs. TRE MEFs ± Dox. n = 180 - 200 nuclei per group. Bars, ratio ± S.E.; *p≤0.05, **p≤0.01, ***p≤0.001; chi-squared test. MEFs where the constraining effects of lamin B1 are no longer present, blebs assume a more random distribution with a significant reduction in polar blebs (Figure 3.8I).

To further test our hypothesis that lamin B1 inhibits the lamin A/C meshwork from protruding outward, we examined lamin B1-overexpressing MEFs (Figure 3.9E). Compared to WT and untreated cells, lamin B1-overexpressing cells had a significantly lower frequency of blebbing nuclei (Figure 3.8B and Figure 3.9F). By increasing lamin B1 expression, we could almost eliminate the formation of nuclear blebs.

3.4 Discussion

Super-resolution localization microscopy is a powerful imaging tool for simultaneous visualization of multiple molecular species at nanometer resolutions (110). Here, we have used STORM combined with quantitative image analysis to identify the spatial organization of A and B-type lamins. We find that the lamin B1 meshwork is closest to the INM and forms a less dense outer rim around the more tightly spaced lamin A/C meshwork facing the nucleoplasm, with a spatial separation of ~15-20 nm. Such precise localization of the two lamin species was achieved by several key technical attributes of our approach. First, it required chromatic aberration-free two-color super-resolution imaging. A complete correction of chromatic aberration is often difficult to achieve, especially when the imaging target is farther away from the coverslip surface (as in our case of imaging equatorial plane of the nuclear periphery). To overcome this hurdle, we used the same reporter dye in the activator-reporter dye pairs in two-color STORM imaging to essentially eliminate any chromatic aberration. Second, by averaging spatial distribution around the nuclear periphery, we can quantify the separation of different lamin types at a higher precision than the

STORM imaging resolution of ~ 20 nm (102). Third, focusing on the equatorial plane maximizes the spatial resolution by imaging the projection of the cross-sectional profiles of the two lamin types across the nuclear periphery.

Our biochemical data confirms the STORM imaging observations that lamin B1 is more closely associated with INM than lamin A/C and that this association is dependent upon the farnesylation of lamin B1. All lamins are initially farnesylated, but only B-type lamins retain their farnesyl group after prelamin A undergoes proteolytic cleavage at its C-terminus (1). This farnesyl tail can allow lamin B1 to tightly associate with the inner nuclear membrane, similar to the way Ras GTPases anchor to the cell membrane (117, 118), and provides a rationale for the retention of the farnesyl group by lamin B1. Interestingly, although the lamin B1 farnesyl mutant significantly altered the lamin B1 network structure, consistent with earlier reports using conventional fluorescence microscopy (23), our results also show that it did not alter lamin A/C distribution, suggesting that proper localization of lamin B1 is not required for the integrity of the lamin A/C network.

While farnesylation of lamin B1's CAAX domain is important for its peripheral localization, other post-translational modifications might also be maintaining nuclear envelope stability. For example, inhibition of Rce1-mediated endoproteolysis or Icmt-mediated carboxymethylation of lamin B1's CAAX domain has been shown to result in compromised structural integrity of the lamina, namely with gaps in the lamin B1 meshwork through which chromatin herniations can be seen (111). Farnesylation of lamin B1 may be critical for its proper localization adjacent to the inner nuclear membrane, but it might not be the only factor. This can now be tested using the STORM imaging protocol we have described.

Taken together with their relative spatial organization at the nuclear periphery (the lamin B1 network overlaying the A-type lamin network), lamin B1 is also less likely to be present in tightly curved structures and is more responsive to bending energy. This implies that the nuclear lamina is composed of independent mechanical elements with distinct properties. Lamin A/C is known to contribute to the mechanical stiffness of the nucleus (103). Other STORM imaging data show that it forms a tighter meshwork that maintains the integrity of the nuclear lamina regardless of shape, as the lamin B1 meshwork has larger inter-filament spaces while being more bending energy responsive (data not included). This complement of stiff and strain-dependent deformable filament structures is not unique to the nuclear envelope. Similar multi-element mechanical complements are also found throughout the cytoskeleton (119).

Although we are lacking data on lamin B2's meshwork in this study, most of the nuclear envelope shape changes can be sufficiently modeled with the physical arrangement of lamin B1's meshwork relative to that of A-type lamins, as well as their mechanical properties. Though knockdown of lamin B2 results in abnormal nucleolar morphology (120), *Lmnb2^{-/-}* mouse fibroblasts do not have significant irregularities in their lamin meshwork (15), and do not exhibit higher frequencies of nuclear blebs than WT fibroblasts (121). It is therefore possible that lamin B2 might contribute very little to nuclear shape dynamics.

Previous studies have shown that individual lamin A and C proteins preferentially homodimerize to form independent but similar mesh-like structures, with little detectable physical interaction with lamin B1 (122-124). Although we have not differentiated between the A- and Ctype lamins in our experiments, our model of the nuclear lamina is not incompatible with this finding, since it is not predicated upon A-type lamins together forming a single, heteromultimeric meshwork at the nuclear periphery. We show that A-type lamins form meshworks that preferentially localize closer to the nucleoplasm, a future direction would be to determine if there are spatial localization differences between lamin A and lamin C networks just as we have observed between lamin A/C and lamin B1, using our sensitive dye pair STORM imaging protocol.

Blebs are often considered to be related to pathological phenomena, especially in the context of laminopathies like HGPS (75) and Emery-Dreifuss Muscular Dystrophy (125), metastasizing cancers (126, 127), or as consequences of nuclear lamina perturbations such as lamin B1 silencing (13). However, we suggest that nuclear blebs are not necessarily pathological abnormalities but may be useful for the rapid nuclear morphology changes needed to respond to intracellular forces such as cytoskeletal contraction. This is supported by our results where a stimulated increase of intracellular forces using Cal-A resulted in significantly increased numbers of blebs. Our data is consistent with a recent report that blebs are also found in wild type cells and these are usually lacking lamin B1 (128). All blebs contain lamin A/C but only a minority also contain lamin B1. In these blebs, lamin B1 is significantly depleted. Together, these results suggest lamin A/C is a major driver of bleb formation. These findings are consistent with another report finding that depletion of all lamins leads to a complete absence of blebs (129). Our micropipette aspiration experiments showed that localization of GFP-lamin B1 and DsRed-lamin A/C along various aspiration lengths is dissimilar to what is observed in naturally occurring blebs. It is important to note that micropipette aspiration induces hyperphysiological strains - high force applied over short times - that allow us to induce energy-dependent deformations independently of cellular forces and visualize lamin network deformation at a rate faster than protein exchange or other biological processes. As such, these shorter time scales and higher forces probably contribute to the distribution of lamin A/C and lamin B1 appearing different than in blebs that occur under normal cellular forces of adherent cells.

We propose that most blebs lack lamin B1 for two main reasons: first, blebs form more readily in the absence of the outer stabilizing layer of lamin B1, and second, because of the curvature dependent localization, lamin B1 is less likely to be located within smaller structures like blebs once they are formed. Whether lamin B1 containing blebs develop from different cellular processes as the blebs containing only A-type lamins remains to be determined. Lamin B1 degradation has been shown to be mediated by autophagy through nucleus-to-cytoplasmic transport of vesicles that deliver lamin B1 to the lysosome (95). It is possible that the lamin B1 containing blebs represent nascent stages of such autophagic vesicles.

We observed a marked difference in bleb orientation among WT and *Lmnb1*^{-/-} MEF nuclei. Nearly 80% of blebs were positioned adjacent to the major axis poles in WT nuclei. With the complete lack of lamin B1, we observed a more random assortment of bleb positioning. In WT cells, the poles are the regions of high curvature and are consequently mostly devoid of lamin B1, while *Lmnb1*^{-/-} cells are uniformly devoid of lamin B1. The blebbing pattern in these two cell types thus mirrors the depleted laminB1 localization and is consistent with our hypothesis that lamin B1 plays a role in suppressing bleb formation in the nuclear envelope. While high curvature strain as a mechanism for preferentially-polar bleb formation as previously proposed still holds (128), our results suggest that this mechanism is also mediated by the absence of lamin B1 at these locations.

In summary, we have identified a novel model for the spatial organization of the nuclear lamina based on two critical principles: 1) Lamin B1 forms a looser, outer meshwork facing the nuclear membrane while lamin A/C forms a tighter, inner meshwork facing the nucleoplasm, and 2) Lamin B1's meshwork is more curvature- and strain-responsive than lamin A/C's meshwork, which affects its localization in tightly curved structures (Figure 3.10).



Figure 3.10: Schematic representation of lamin organization at the nuclear envelope.

This model reveals a role for lamin B1 in preventing the A-type lamin meshwork from protruding outward and can predict the disparate structural consequences of perturbing individual lamina components. However, this model does not explain the connection between lamin B1 and myelination. For that, aim 2 explores the role of lamin B1 in developing and mature oligodendrocytes.

[Note: The data from this chapter has been published in: Nmezi B, Xu J, Fu R, Armiger TJ, Rodriguez-Bey G, Powell JS, Ma H, Sullivan M, Tu Y, Chen NY, Young SG, Stolz DB, Dahl KN, Liu Y, Padiath QS. *Concentric organization of A- and B-type lamins predicts their distinct roles in the spatial organization and stability of the nuclear lamina*. Proceedings of the National Academy of Sciences. 2019;116(10):4307.]

In animal cell nuclei, lamin B1's meshwork (green) lies closest to the inner nuclear membrane, while lamin A/C's meshwork (red) faces the nucleoplasm. (A) In circular or slightly elliptical nuclei, the B-type lamin meshwork is sufficient to contain the underlying A-type lamin meshwork. (B) Regions of high curvature in elongated nuclei can result in a dilation or loss of the lamin B1 meshwork. (C) Lamin A/C forms blebs through the gaps within the lamin B1 meshwork. Representative MEF nuclei displaying (D) circular, (E) elongated, and (F) blebbed morphologies as illustrated in the above models. Lamin B1 (green), lamin A/C (red), DNA (blue).

4.0 The Role of Lamin B1 in Mouse Oligodendrocyte Development and Proliferation

4.1 Introduction

The fact that ADLD pathophysiology exclusively consists of neurological symptoms despite a germline duplication of LMNB1, present in all cell types, suggests the possibility that lamin B1 has a special role in the nervous system. A routinely used method for discovering the roles of proteins *in vivo* is to generate knockout models of the gene in question in mice. Complete lamin B1-deficient mice are perinatal lethal (9), so tissue-specific conditional knockout models are required to study the role of lamin B1 in brain development. Previous research has found that lamin B1 in neurons is required for proper neuronal development and migration, as neuron-specific lamin B1-knockout mice exhibited impaired brain development and aberrant neuronal morphology (11, 23). However, no changes in myelination, the hallmark of ADLD, have been described. Therefore, lamin B1 may also play an important role within other cells in the CNS – namely oligodendrocytes (OLs), the cells responsible for generating myelin. Previously published data showed that mouse OLs are very susceptible to overexpression of lamin B1 (80), leading to age-dependent ADLDlike symptoms, so OL-specific Lmnb1 knockout mice should give us more insights into the roles of lamin B1 in myelination. To accomplish this, I utilized cre-lox recombination system to knock out *Lmnb1* specifically in mouse OLs. The cre recombinase gene is under the transcriptional control of the OL-specific Cnp promoter, while the mouse Lmnbl gene is flanked by loxP sequences (Lmnb1-flox). Double-transgenic mice hemizygous for Cnp-cre and homozygous for floxed *Lmnb1* have *Lmnb1* knocked out in OL lineage cells.

4.2 Materials and Methods

4.2.1 Generation of conditional knockout transgenic mice

Hemizygous *Cnp-cre* mice (Lappe-Siefke et al. (56)) were mated to homozygous *Lmnb1*^{n/n}</sub> mice (Yang et al. (12), Jackson Laboratories #032558), on a C57BL/6J background. The resulting*Cnp-Cre;Lmnb1*^{<math>n/n}</sub> mice were again crossed to*Lmnb1*^{<math>n/n} ("Wt") mice in order to get *Cnp-Cre;Lmnb1*^{n/n}</sup> (conditional knockout, "cKO") mice. Further crosses were done using*Cnp-Cre;Lmnb1*^{<math>n/n}</sup> (conditional knockout, "cKO") mice. Further crosses were done using*Cnp-Cre;Lmnb1*^{<math>n/n}</sup> x*Lmnb1*^{<math>n/n} in order to obtain 50% Wt and 50% cKO mice per litter. All cKO mice were hemizygous for the *Cnp-Cre* transgene. Wt littermates were used as controls for their cKO counterparts, and mice of either sex were used in experiments. For tamoxifen injection of *UBC-Cre-ERT2* mice (Rusankina et al. (131), Jackson Laboratories #008085), tamoxifen (Sigma) was dissolved in corn oil (Sigma) at 20 mg/mL, and administered via IP injection at approximately 75 mg/kg body weight every 24 hours for 5 consecutive days. All animals were housed in University of Pittsburgh animal facilities managed by the Division of Laboratory Animal Resources in accordance with Institutional Animal Care and Use Committee guidelines.</sup></sup></sup></sup></sup></sup></sup>

4.2.2 Mouse genotyping

Ear punches were used to genotype mice 20 days and older. For mice under 20 days of age, 1-2 mm of tail snips were used. Ear punches and tail snips were digested in DirectPCR Tail Lysis Buffer supplemented with 200 μ g/mL proteinase K (Viagen) overnight at 55°C. Samples were centrifuged at 12,000xg for 1 minute and 2 μ L of supernatant was used for genotyping PCR. Genomic DNA used in PCR was diluted to 50 ng/uL working concentration, and 100 ng DNA was used per reaction. Genotyping primer sequences are listed in Appendix B.

4.2.3 Open field activity monitoring test

Activity monitoring tests were performed in the Rodent Behavior Analysis Core at the University of Pittsburgh. Tests were performed in controlled conditions during the day phase of the day/night cycle. 5 Wt and 5 cKO mice at 5 months old were used for testing. Mice were individually placed into an open-field apparatus (43 x 43 x 30 cm; Med Associates) and allowed to freely explore for 30 minutes. Horizontal and vertical movements were monitored with infrared light beams and detectors. Beam interruptions were analyzed with custom software (Med Associates) that measure ambulation, velocity, distance traveled, and rearing.

4.2.4 Tissue dissection and processing

For genomic DNA, RNA, and protein isolation, mice were euthanized with CO₂ inhalation and the brain and cervical spinal cord were dissected over cold 1x PBS. Brains were cut in half, separating the left/right hemispheres, then further separated into forebrain, brainstem, and cerebellum. Cervical spinal cords were cut to separate proximal/distal halves. All samples were then snap frozen in liquid nitrogen and stored at -80°C until ready for processing. CKO samples and their corresponding Wt controls were always taken on the same day. For cell proliferation, animals at postnatal day 5 were injected with 100 µg/kg-bodyweight 5-ethynyl-2'-deoxyuridine in PBS, three times with 4 hours between each injection, and perfused 24 hours after time of first injection. For immunostaining, mice were anesthetized with intraperitoneal injection of 2.5% 2,2,2-tribromoethanol (Sigma), then transcardial perfusion was performed with ice-cold 1x PBS followed by ice-cold 4% formaldehyde in PBS. Brain and spinal cord were extracted then postfixed overnight with 4% formaldehyde in PBS, followed by cryoprotection with 30% sucrose in PBS at 4°C. Samples were embedded in OCT (Fisher) over dry ice, and 10 µm cryosections were taken for immunostaining on Superfrost Plus slides (Fisher). For EM analysis, mice were transcardially perfused with ice-cold 1x PBS followed by 4% formaldehyde and 2.5% glutaraldehyde in PBS. For TEM analysis, specimens were processed at the University of Pittsburgh Center for Biologic Imaging. The specimens were rinsed in PBS, post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) with 1% potassium ferricyanide (Fisher), dehydrated through a graded series of ethanol (30% - 100%, Fisher) and propylene oxide (Electron Microscopy Sciences), and embedded in Poly/Bed® 812 (Luft formulations) and Dimethylaminomethyl (Polysciences). Semi-thin (300 nm) sections were cut on a Leica Reichart Ultracut (Leica Microsystems), stained with 0.5% Toluidine Blue in 1% sodium borate (Fisher) and examined under the light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate (Electron Microscopy Sciences) and Reynold's lead citrate (Fisher) and examined on JEOL 1011 transmission electron microscope (JEOL, grant #1S10RR019003-01 NIH for Simon Watkins) with a side mount AMT 2k digital camera (Advanced Microscopy Techniques).

4.2.5 Genomic DNA extraction

Frozen mouse brain samples were crushed in a ceramic mortar and pestle over dry ice and ~100 mg were incubated in cell lysis solution (Qiagen) with proteinase K (Viagen) overnight at 55°C. Genomic DNA extraction was carried out using the Gentra Puregene kit (Qiagen) according to the manufacturer's instructions.

4.2.6 RNA extraction and real-time PCR

Samples were homogenized in TRIzol (Invitrogen) using a Dounce homogenizer (Kimble), and extracted according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA using qScript cDNA Synthesis Kit (Quanta Bio). Real-time PCR was performed using PerfeCTa SYBR Green SuperMix with ROX (Quanta Bio) on an ABI QuantStudio 12K Flex (Applied Biosystems). Data were analyzed using the $\Delta\Delta C_T$ method (132), using mouse β-actin (*Actb*) as an endogenous control. Real-time PCR primer sequences are listed in Appendix B.

4.2.7 Western blotting

Protein lysates from brain and spinal cord were obtained by homogenizing them in T-PER supplemented with 1x protease inhibitor cocktail (Thermo Scientific). Protein concentration was measures by BCA protein assay kit (Thermo Scientific). PAGE was performed with up to 50 µg total protein in each well of a 10% polyacrylamide gel (Bio-Rad), and proteins were transferred to nitrocellulose membranes (Bio-Rad), followed by blocking with 4% nonfat milk in TBS or 1x Odyssey blocking buffer (LI-COR Biosciences) for 1 hour at room temperature. Membranes were incubated with primary antibodies diluted in 5% nonfat milk in TBS or 1x Odyssey blocking buffer (With 0.1% Tween-20 (Fisher) overnight at 4°C with gentle shaking. Primary antibodies used and their dilutions are listed in Appendix A. The next morning, membranes were washed three times for 10 minutes each with TBS-T (TBS + 0.1% Tween 20), and incubated with goat anti-mouse 680LT (LI-COR #926-68020) and donkey anti-rabbit 800CW (LI-COR #926-32213) secondary antibodies diluted 1:10000 in Odyssey blocking buffer + 0.1% Tween 20 for 1 hour at room temperature in the dark. After three 10-minute washes in TBS-T, membranes were scanned,

imaged, and quantified using the LI-COR Odyssey CLx infrared scanner and Image Studio software (LI-COR Biosciences).

4.2.8 Immunofluorescence staining

Sections on slides were circled with a PAP pen to form a hydrophobic barrier, and were blocked using 5% normal donkey serum (Jackson ImmunoResearch) + 0.3% Triton X-100 (Sigma) in PBS for 1 hour at room temperature. Primary antibodies were diluted in PBS + 1% BSA (Sigma) + 0.3% Triton X-100 and incubated with the cells overnight at 4°C. Primary antibodies used and their dilutions are listed in Appendix A. The next morning, primary antibody dilutions were discarded and slides were washed with PBS three times for 5 minutes each at room temperature. FITC conjugated donkey anti-rabbit (Jackson ImmunoResearch #711-095-152) and Cy3 conjugated donkey anti-mouse (Jackson ImmunoResearch #715-165-150) secondary antibodies were each diluted 1:350 in PBS + 1% BSA, and incubated with the sections for 1 hour at room temperature in the dark. Slides were washed three times for 5 minutes each in PBS, then mounted with 24 x 50 mm glass coverslips (Corning) and Vectashield antifade mounting medium with DAPI (Vector Laboratories).

4.2.9 Imaging and quantification

The following imaging was done in the Padiath lab (Department of Human Genetics): Wide-field microscopy images were acquired of immunofluorescently-labeled tissues on a Leica DM5000B upright microscope and Leica DFC310 FX digital camera (Leica). Five to seven nonoverlapping fields across corpora callosa (medial to lateral) were quantified per brain section, with four 10 µm sections from each mouse representing approximately 0.5 mm from the bregma (rostral to caudal) using 20x and 40x objectives. Counts were done via imageJ (FIJI) using regular counting analysis, counting cells per corpus callosum field. Results were graphed as the average of counts per field per mouse.

4.2.10 Statistical analysis

Two-sided *t*-tests were used to calculate statistical significance between two groups. The following convention for representing p values was followed: $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$. A p-value ≤ 0.05 was considered statistically significant. All error bars represent standard error unless otherwise specified. Data were graphed and analyzed in Microsoft Excel 2016 and GraphPad Prism 7.

4.3 Results

4.3.1 Lmnb1 is partially deleted in conditional knockout mice

To study the effect that deletion of *Lmnb1* has on the development of OLs, I used previously generated transgenic mice expressing Cre recombinase under the control of the *Cnp* promoter and mated them into lines with homozygous floxed *Lmnb1*. *Cnp1* codes for 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (Cnpase), a myelin-associated enzyme (133) whose promoter sequence has been previously used to target gene expression in mouse OLs (134). To confirm that *Lmnb1* is being deleted in conditional knockout (cKO) mice, I designed primers surrounding the

loxP sequences flanking exon 2 in *Lmnb1*-flox mice to test specificity and effectiveness of recombination (Figure 4.1A). Amplification of floxed *Lmnb1* results in a 2.1 kb fragment, compared to Wt (non-floxed) Lmnb1's 1.35 kb fragment. The removal of exon 2 by cre recombinase results in an 850 bp PCR product. The PCR shows the deleted *Lmnb1* in band in cKO mice is specific to DNA extracted from their CNS and not from ear punches, thanks to the specificity of the *Cnp* promoter (Figure 4.1B). As a positive PCR control for *Lmnb1* deletion, I have used genomic DNA from *Lmnb1*-flox mice also carrying *UBC-Cre-ERT2* injected with tamoxifen (131). Note that there is still a faint PCR product corresponding to uncut floxed *Lmnb1* in the cKO brain. Since not all cells in the brain are of OL lineage, a population would not express the *Cnp-cre* transgene and would therefore show the uncut floxed *Lmnb1* PCR product. The smaller PCR band corresponding to the deleted *Lmnb1* gene is preferentially amplified in PCR, therefore it does not accurately represent the ratio of recombined to un-recombined DNA.



Figure 4.1: Characterization of Lmnb1 cKO mouse knockouts.

(A) Mechanism of action (not to scale). Cre recombinase excises *Lmnb1* exon 2, which is flanked by loxP sequences. "For" and "Rev" designate primers used to confirm genomic DNA deletion of exon 2 after recombination. (B) PCR confirmation of brain-specific excision. Genomic DNA from cerebellum (CB) and ear punch of a *Cnp-Cre;Lmnb1*^{*fl/fl*} mouse was PCR amplified along with ear punch from a *UBC-Cre;Lmnb1*^{*fl/fl*} and CB samples from one *Lmnb1*^{*fl/fl*} and two Wt mice. Expected sizes: *Lmnb1-flox* = 2.1 kb, *Lmnb1-Wt* = 1.35 kb, *Lmnb1-Δ* = 840 bp. (C) *Lmnb1* mRNA and (D) protein expression is mildly reduced in cKO whole forebrain (FB) compared to Wt samples. Graphs are average \pm SEM, n = 3.

Total mRNA and protein analyses of mouse forebrain show a small but statistically insignificant reduction of lamin B1 (Figure 4.1C and D). This is because the brain is composed of a diverse population of cells, only a small portion of which are of OL lineage. Therefore, not all cells within the forebrain will express the *Cnp-Cre* transgene, and any reductions in OLs may be masked by lamin B1 expression from various other cell types. Since it is challenging to find measurable expression differences of lamin B1 protein and mRNA from total brain tissue, immunofluorescence microscopy was used to analyze lamin B1 expression changes within OL lineage cells of the corpus callosum. We focused on the corpus callosum as it is a well-defined white matter (i.e. myelinated) region. Analysis of Olig2, a transcription factor that marks all OLlineage cells, and lamin B1 co-staining in P6, P15, and P30 mouse corpora callosa (Figure 4.2A-D) show a significant decrease of Olig2 and lamin B1 double-positive cells in cKO versus Wt sections at P15 and P30, but not at P6 (Figure 4.2E). The *Cnp-Cre* transgene is not activated early enough (by P6) to eliminate Lmnb1 expression completely from progenitor cells (134). Since lamin B1 is a long-lived protein (135), the lamin B1 that we still see in OLs may be previously-generated protein that has yet to be degraded.



Figure 4.2: Lamin B1 is reduced in cKO brains at P15 and P30, but not P6.

Representative images of (A) P15 Wt, (B) P6 cKO, (C) P15 cKO, and (D) P30 cKO corpus callosum stained for Olig2 and lamin B1. Arrows point to examples of Olig2+ cells that are also lamin B1+. (E) Counts of Olig2+ cells that also contain lamin B1 show that cKO mice have a significant decrease of lamin B1 in oligodendrocytes at P15 and P30 but not at P6. Graphs are average \pm SEM, n = 3, ** = p < 0.01.

4.3.2 No differences in activity or brain sizes between cKO and Wt mice

At all ages, cKO mice were physically indistinguishable from their Wt counterparts. Openfield activity tests in adult mice show no differences between Wt and cKO mice in all measured characteristics (Figure 4.3A). Whole brains from cKO mice also did not appear grossly altered, being the same size as brains from Wt mice at all ages, from 15 days old to 1 year (Figure 4.3B).



Figure 4.3: There are no discernible physical differences between Wt and cKO mice.

(A) Quantification of 30 minute open field activity monitoring of 5 month old mice. n = 6. (B) Size comparisons of perfused and formaldehyde-fixed brains from P15 (left) and 12 month old (right) Wt and cKO mice, showing no differences in size or development.

4.3.3 Myelin gene expression is unchanged in cKO mice



Figure 4.4: Reduction of Lmnb1 in mouse oligodendrocytes.

Lmnb1 reductions in mouse mature oligodendrocytes does not result in large myelin protein expression expression changes in mouse forebrain. Cnpase is reduced by 50% in all cKO animals due to the knock-in of Cre transgene. Graphs are fold relative Wt control \pm SEM. n = 3 biological replicates, * = p<0.05, ** = p<0.01, *** = p<0.001.

Real-time PCR analysis of myelin genes P*lp*, *Mbp*, *Mog*, and *Mag* in mouse forebrain samples show mostly no significant differences in Wt versus cKO at all time points (Figure 4.4A). P30 cKO mRNA had slightly higher expression of *Mbp* and *Mog* that were statistically significant, but the differences were slight. Western blot analyses of myelin proteins show no differences in PLP and MBP expression within Wt versus cKO samples at all time points (Figure 4.4B). However, we do see reductions of MOG and MAG protein in cKO mice. It is unclear if these reductions are physiologically relevant, considering they do not affect mouse activity or survival into adulthood. In all cKO mice at all timepoints, Cnpase protein and mRNA expression is reduced by approximately half. This is not due to the knockout of *Lmnb1*, but because the insert of the transgenic Cre recombinase gene into the mouse *Cnp* locus via knock-in thus inactivating one copy of the gene (56). Previous research using these mice as a heterozygous knockout model of Cnpase did not find differences in survival or behavior compared to Wt mice up to 1 year of age (56, 136).

4.3.4 Alterations in numbers of OL lineage cells in cKO mice

Although we did not observe any overt alterations in the levels of myelin proteins, as lamin B1 is known to play a role in cellular proliferation and differentiation, we sought to determine whether loss of lamin B1 can impact the number and maturity of OL lineage cells in the corpus callosum using immunohistochemistry (IHC). To identify OLs in different stages of maturation, we used markers that have been previously shown to be specific for each stage. The OL-specific transcription factor Olig2 is present in all OL lineages irrespective of their maturation stage. PDGFR α is expressed by oligodendrocyte progenitor cells (OPCs) while CC1 is expressed by mature OLs (137-140). PDGFR α +Olig2 double labeling thus marks OPCs while CC1+Olig2 labeling marks mature OLs.

PDGFR α +Olig2 staining did not show significant differences between Wt and cKO mouse brains at P15 (Figure 4.5A and B), but CC1+Olig2 staining showed significant decreases at both P15 and P30 (Figure 4.5C and D). This would suggest that OPCs lacking lamin B1 have defects in their ability to differentiate into mature OLs.


Figure 4.5: cKO mice OPCs have differentiation defects.

(A) Wt vs. cKO P15 brain sections stained against Olig2 (red) and PDGFR α (green). Arrows point to examples of cells that are positive for both. (B) Quantification of PDGFR α +Olig2 double-positive cell counts in mouse corpus callosum at P15 and P30. There are no differences between Wt and cKO mice. (C) Wt vs. cKO P15 brain sections stained against Olig2 (red) and CC1 (green). Arrows point to cells positive for both. (D) Quantification of CC1+Olig2 double-positive cell counts in mouse corpus callosum at P15 and P30, showing a reduction of CC1+ cells in cKO brains. Graphs are average \pm SEM. n = 3 biological replicates, ** = p<0.01, *** = p<0.001.

If this were indeed the case, we would expect a reduced number of mature OLs that expressed lamin B1. We therefore measured the colocalization of lamin B1 with CC1 (Figure 4.6A and B). Consistent with data from Figure 4.5, we found that cKO mice have fewer CC1+ cells at P15 and P30 (Figure 4.6C), but there are also significantly fewer CC1 and lamin B1 doublepositive cells in cKO mice than in Wt (Figure 4.6D). This would suggest that OPCs lacking lamin B1 are defective in their ability to differentiate into mature OLs. Although we would expect to see no lamin B1 in mature OLs, it is possible that small population of OPCs that escape the excision of the lamin B1 locus would preferentially differentiate into mature OLs and therefore supplant the OLs lacking lamin B1. The fact that we can observe mature OLs without lamin B1 would suggest that OPCs have maturation difficulties without lamin B1, but they are also able to survive once matured. This is consistent with previous data demonstrating that lamin B1 levels are significantly reduced in mature OLs (141).



Figure 4.6: There are fewer mature oligodendrocytes with lamin B1 in cKO mouse brains.

Immunofluorescent staining of (A) Wt and (B) cKO mice against lamin B1 (green) and CC1 (red) in the medial corpus callosum. Arrows point to CC1+ cells that are negative for lamin B1. (C) Counts of CC1+ cells per field show there are fewer mature oligodendrocytes in cKO brains at P15, with a large difference at P30. (D) counts of CC1+lamin B1 double-positive cells per field show there are fewer mature oligodendrocytes with lamin B1 in cKO brains. Graphs are average \pm SEM. n = 3 biological replicates, * = p<0.05, ** = p<0.01, *** = p<0.001.

To determine whether there were proliferation defects in OL lineages in the cKO mice, we measured 5-ethynyl-2'-deoxyuridine (EdU) staining, a nuclear marker for proliferating cells. Mice were injected with thymidine analog EdU at P6 and P15, and Olig2+EdU cells in the corpus callosum were stained and counted. P6 brains had a higher proportion of replicating OLs than P15 brains in general, but no significant differences were seen between Wt and cKO brains (Figure 4.7A). However, EdU is only incorporated into cells at the S-phase of the mitotic process, consequently underestimating the number of dividing cells in the CNS (142). Other proliferation markers such as Ki-67 should be used to investigate OL proliferation in future studies. To confirm that the reduction in the number of mature OLs in the cKO mice was not due to apoptosis, we carried out staining for activated caspase 3, a cytoplasmic marker for apoptotic cells, across all time points (Figure 4.7B). No Olig2+ cells that were also positive for activated caspase 3 were observed in corpora callosa of Wt or cKO mice.



Figure 4.7: It is unclear if cKO oligodendrocytes have proliferation defects.

(A) Proportion of Olig2+ cells that are EdU+ 24 hours after IP injection of EdU is very small in both Wt and cKO mice. There are no significant differences between Wt and cKO mice. Graphs are average \pm SEM, n = 3. (B) Representative immunofluorescence staining of Olig2 (red, arrowheads) and activated caspase 3 (green, arrow) in the caudate putamen of a P15 mouse. No Olig2+ cells had activated caspase 3 staining at P15, P30, or 3 months in the corpus callosum or other imaged sections of the brain.

4.4 Discussion

Our results indicate that the loss of lamin B1 in OL lineages leads to a reduction in the number of mature OLs. Given that we did not observe an alteration in OPC numbers in the cKO mice, this would indicate that lamin B1 lacking OPCs are defective in their subsequent ability to differentiate into mature OLs.

We did not observe any obvious functional defects in the expression of myelin proteins nor in the gross morphology of the brain or of the corpus callosum, the major white matter tract that we have studied. Expression of *Plp*, *Mbp*, *Mog*, *Mag* or their protein products are minimally altered in the forebrains of cKO mice. At 5 months, cKO mice do not exhibit activity changes. Mature cKO mice are also physically indistinguishable from their Wt littermates up to at least one year of age. While this could suggest that lamin B1-lacking OLs are functionally normal, this explanation must be qualified by a few caveats. We observed a significant number of mature OLs (~ 50%) that did exhibit lamin B1 expression. We reasoned that these represented the progeny of a small population of OPCs that escaped excision by the cre recombinase but that preferentially matured into OLs. In addition, Lamin B1 is also known to be an extremely long-lived protein. Thus, even if the lamin B1 locus has been excised in the late OPC and early OL stages, the residual lamin B1 from OPCs could still remain and account for the lamin B1 positive OLs we have observed.

The relatively normal myelination profile could therefore be due to these populations of OLs that still retain lamin B1 and would mask any functional defect due to the lamin B1 lacking OLs. The *Cnp* promoter is thought to express in the late OPC stage and become strongest in mature OLs. This is consistent with our observation that *Lmnb1* is not knocked out at P6 and exhibits a maximal reduction in expression only at later time points (53). While our results point to a defect in the differentiation from OPCs to OLs as the cause of the reduction in the number of mature OLs

in the cKO mice, it would be premature to conclude that it does not have any effect on the proliferation of OPCs. It is important to remember that mature OLs lose their ability to replicate, and that proliferation only occurs in OPCs. Thus, we would ideally want to ablate lamin B1 in the early OPC stage using a different promoter such as *Olig2* (143). Experiments using *Olig2-Cre* mice crossed to lamin B1 floxed mice are now underway, which would allow us to more clearly define the role of lamin B1 in OPCs and determine whether the reduction in mature OLs we observe is also not due to a replication defect. We also aim to measure ultrastructural changes to myelination in young and old mice.

Considering that the consequences of excess lamin B1 protein in mature OLs lead to severe demyelination (80, 144), lamin B1 expression is tightly regulated in mature OLs for their proper function (145). Mature OLs may fare well with *Lmnb1* knockout because its expression is already meant to be downregulated at that stage. Future studies into lamin B1 regulation in OLs and white matter has interesting implications with respect to demyelinating diseases such as ADLD: there may be OL-specific regulatory mechanisms of lamin B1 found in humans. Aim 3 further explores this possibility in detail.

5.0 Genomic Rearrangements Involving LMNB1 and ADLD Pathogenesis

5.1 Introduction

In my second aim, I established that a knockout of lamin B1 in mouse OLs *in vivo* does not result in obvious morphological or behavioral deficiencies, so mature OLs may be able to function normally with minimal lamin B1. On the other hand, OLs are very susceptible to increased lamin B1 levels, as its overexpression leads to severe ADLD-like symptoms in mice (80). For this reason, OLs may employ unique mechanisms for reducing lamin B1 expression once matured.

Canonically, head-to-tail tandem genomic duplications of *LMNB1* cause ADLD (77). It is thought that an extra copy of *LMNB1* is sufficient to cause its overexpression thus leading to the classic ADLD phenotype. As it turns out, this is not always the case. Previous research has described a family with ADLD that did not have duplication of *LMNB1* (88). Instead, this family has a large genomic deletion upstream of *LMNB1*. It is currently hypothesized that lamin B1 overexpression in this case is caused by the deletion of a boundary that separates one topologically associating domain (TAD) from another. This deletion merges *LMNB1* and upstream enhancers into the same TAD, presumably leading to the enhancers inducing lamin B1 overexpression. However, this mechanism does not sufficiently explain other CNVs in the pathogenesis of ADLD. For example, I have found that not all *LMNB1* duplications lead to ADLD. In this chapter, I will describe families that have non-recurrent genomic duplications surrounding *LMNB1* but lack demyelinating disorders. Understanding how CNVs surrounding *LMNB1* lead to disease could be the key to understanding how *LMNB1* is regulated within OLs and white matter.

Here, I have used array-based comparative genomic hybridization (aCGH) on DNA from patients with ADLD or autonomic dysfunction to correlate their copy number variants in 5q23.2 to their clinical outcomes. I propose a novel OL-specific cis-regulatory element that silences *LMNB1* expression in white matter. The existence of such an element would offer a comprehensive model linking copy number variants surrounding *LMNB1* with ADLD pathogenesis.

5.2 Materials and Methods

5.2.1 Collection of clinical data, DNA isolation, and array CGH

All clinical evaluations took place at their respective institutions, under the guidelines of their institutional review boards. All participants provided written informed consent. Genomic DNA from whole blood, saliva, primary fibroblasts and brain tissue was isolated using the Gentra Puregene kit (Qiagen) according to the manufacturer's instructions. aCGH was performed at the University of Pittsburgh on genomic DNA from clinical samples, hybridized a custom 8x15K HD-CGH microarray, scanned in a G2565CA microarray scanner, and analyzed using Agilent CGH Analytics software (Agilent Technologies) according to the manufacturer's instructions. Human genome assembly GRCh37/hg19 was used for all genome coordinates. PCR primers were designed to specifically amplify the tandem duplication junctions for each family using Longamp Taq Polymerase (New England Biolabs), then Sanger sequenced (Eurofins Genomics) after treatment with ExoSAP-IT reagent (Applied Biosystems).

5.2.2 Primary fibroblast isolation and cell culture

Patient and control skin biopsies were minced to smaller than 1.0 mm³ pieces then incubated with 0.05% Trypsin-EDTA (Sigma) for 3 hours at 37°C with gentle mixing. Pieces were then plated on a 10-cm tissue culture dish (ThermoFisher) with a minimal amount of DMEM complete (high glucose DMEM (Corning) supplemented with 10% FBS (Fisher), 2mM Lglutamine (Millipore), and 1% penicillin-streptomycin (Hyclone)) until they adhered to the culture dish. Primary fibroblasts were trypsinized and re-plated into new dishes as they exited the explant.

5.2.3 RNA isolation and real-time PCR

RNA was isolated from cells and brain tissue using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from 1 µg of RNA using qScript cDNA Synthesis Kit (Quanta Bio). Real-time PCR was performed using PerfeCTa SYBR Green SuperMix with ROX (Quanta Bio) on an ABI QuantStudio 12K Flex (Applied Biosystems). Data were analyzed using the $\Delta\Delta C_T$ method using human β-actin (*ACTB*) as an endogenous control. Primer efficiencies were validated using 6-fold serial dilutions of cDNA. Real-time PCR primer sequences are listed in Appendix B.

5.2.4 SNP height analysis

Primers were designed to amplify the 3' untranslated region (UTR) of the human *LMNB1* gene encompassing SNPs rs1051643 and rs1051644. Primer sequences are listed in Appendix B. PCR products were treated with ExoSAP-IT reagent (Applied Biosystems) then Sanger sequenced

(Eurofins Genomics). Sequences and electropherograms were analyzed using Sequencher v. 5.4 software (Gene Codes).

5.3 Results

5.3.1 Genomic deletions upstream of LMNB1 lead to ADLD

Patients from three unrelated families (DEL1, DEL2, and DEL3) had ADLD, but were negative for mutations in known disease-causing genes including duplications of *LMNB1*. A custom aCGH assay was used to map copy number variants surrounding *LMNB1* on 5q23.2, revealing large heterozygous deletions from ~250 kb to ~670 kb extending to within ~4.8 kb to 88 kb upstream of *LMNB1* (Figure 5.1). Analysis of the deletion boundaries revealed that all boundaries lie within highly repetitive elements such as *Alu* repeats and long interspersed nucleotide elements (LINEs) (Table 3.1). It is likely that genomic deletions occur through non-allelic homologous recombination or microhomology-mediated end joining (146). Deletion events were also clearly non-recurrent: each family has a unique deletion upstream of *LMNB1*.

Table 5.1: Details of ADLD deletion families

Family ID	Centromeric junction (chr5)	Telomeric junction (chr5)	Deletion size (kb)	Centromeric repeat	Telomeric repeat	Microhomology at junctions
DEL1	125857895	126108379	250	Alujr	AluSp	None
DEL2	125352465	126024979	673	10bp LINE homology	LINE (LIMC5)	None
DEL3	125492412	126101296	609	AluSg7	AluSP	26 bp

Using aCGH data, I identified the "critical deletion region" ("Del Crit") from the overlap of all identified deletion coordinates. Del Crit extends from chr5:125857895-126024979 (Figure 5.1) and encompasses three genes: *ALDH7A1*, *PHAX*, and *TEX43*; as well as a boundary between two topologically associating domains (TADs) previously determined by chromosome conformation capture (86, 147), separating *LMNB1* from previously-identified enhancer elements upstream of *LMNB1* (88). It is possible that the deletion of this TAD boundary would result in aberrant interactions between *LMNB1* and these upstream enhancers. While this remains plausible, another mechanism of action may be involved, which will be explored next.



Figure 5.1: Schematic representation of upstream deletions that lead to ADLD.

Blue bars represent deleted regions. DEL1, DEL2, and DEL3 are unrelated families with ADLD. "Enh" refers to a putative forebrain-specific enhancer element [83]. The minimal overlapping region of all deletions is defined as the critical deletion region ("Del Crit") which includes *PHAX*, *ALDH7A1*, *TEX43* (not pictured), and a topologically associated domain boundary.

5.3.2 Large genomic duplications surrounding LMNB1 do not cause ADLD

Patients from three unrelated families (K9, K11, and Port) were found to have mild autonomic disorders with an autosomal dominant inheritance pattern. Custom aCGH analysis in 5q23.2 found a heterozygous duplication involving the *LMNB1* gene, but intriguingly, they showed no symptoms of ADLD and Magnetic Resonance Imaging (MRI) revealed no signs of demyelination. This was true even in patients >60 years of age, by which time patients with ADLD show significant MRI alterations, are profoundly debilitated by limb weakness, and in some cases paralysis (148, 149). Their genomic duplications include not only *LMNB1*, but a significant portion of the upstream sequences as well. Duplication sizes range from ~466 kb to over 4.3 Mb (Figure 5.2A), much larger than the typical duplication sizes found in ADLD patients: ~150 kb to ~200 kb (81). Sequencing analysis revealed that patients have simple tandem duplications with repetitive elements surrounding most of the duplication breakpoints (Table 3.2), therefore these duplications are likely caused by replication-based mechanisms such as microhomology-mediated break-induced replication (MMBIR) (83).

Family ID	Centromeric junction (chr5)	Telomeric junction (chr5)	Duplication size (kb)	Centromeric repeat	Telomeric repeat	Microhomology at junctions
P1	125253821	126285172	1,031,351	LTR: HERVP71A	None	None
K9	125847699	126314321	466,623	SINE: AluSx	LTR: Charlie4z	AG
K11	121812949	126178948	4,366,000	LTR: HERVH	LTR: HERVH	49 bp
BR1 Ref. [81]	125699519	126174517	474,998	LINE: L1MB7	None	Complex

Table 5.2: Details of large duplication families

However, not all families with large duplications are spared from demyelination. Previously identified family "BR1" (81) has a ~475 kb heterozygous duplication surrounding *LMNB1*, and affected members have very severe ADLD symptoms with much earlier ages of onset (their twenties instead of forties to fifties). This duplication is also unique in its complexity: instead of having a simple tandem duplication like other families with ADLD, BR1's duplicated copy is inverted and inserted just upstream of *LMNB1* (Figure 5.2B).



Figure 5.2: Schematic representation of large genomic duplications involving LMNB1.

(A) Green bars represent duplicated regions. K11, Port, and K9 are families identified in this study that have *LMNB1* duplications but no ADLD. BR1 is a previously-identified family (Giorgio et al. 2013, [81]) with a severe form of ADLD. The large duplication critical region "LD Crit," the overlap of all known large duplications, defines the minimal duplicated region that includes *LMNB1* but does not lead to ADLD. (B) Schematic representation of typical CNVs surrounding *LMNB1* and their ADLD outcomes. The duplication within BR1 family is inverted and inserted 5' of *LMNB1*'s transcription start site.

Our data so far suggest that the canonical model of ADLD etiology – a simple tandem duplication of LMNB1 – is no longer sufficient to explain the mechanisms of LMNB1 overexpression. Patients lacking LMNB1 duplications may still develop ADLD, while some with duplicated LMNB1 do not. I define the "critical large duplication region" ("LD Crit") as the

minimal overlapping region of all known *LMNB1* duplications without ADLD (therefore excluding BR1). LD Crit extends from chr5:125847699-126285172 (Figure 5.2A), completely encompassing Del Crit, suggesting that *LMNB1* may be regulated through an element present within Del Crit that is excluded from all ADLD-causing duplications. Such a regulatory element may exhibit tissue specificity: tissues with highly proliferative cells may require consistent lamin B1 expression, while others with non-proliferative cells (e.g. mature oligodendrocytes) may require lower lamin B1 expression. Expression of *LMNB1* should therefore be analyzed in multiple tissue types.

5.3.3 LMNB1 expression in ADLD is highest within brain white matter

RNA from primary fibroblasts derived from ADLD patient and control skin biopsies were isolated and analyzed for *LMNB1* expression. Brain samples from two unrelated individuals with ADLD, with separate sections from grey matter (containing mostly cell bodies and few myelinated axons) and white matter (containing a high density of myelinated axons and oligodendrocyte cell bodies), were also analyzed for *LMNB1* expression. mRNA analysis in fibroblasts and grey matter from patients with classical ADLD duplications showed moderately increased *LMNB1* transcript with respect to control samples, consistent with previous data from patient lymphoblasts (150). However, duplication ADLD patients had significantly higher *LMNB1* expression in white matter compared to controls (Figure 5.3A). ADLD patients with upstream deletions did not have increased *LMNB1* mRNA in fibroblasts, but significantly increased *LMNB1* expression in white matter (Figure 5.3B). Grey matter samples from deletion ADLD brains were not available. *LMNB1* expression in white matter was much higher than in fibroblasts or grey matter within all ADLD patients, indicating a white matter-specific mechanism of *LMNB1* overexpression, independent

from copy number. Fibroblasts from large duplication family members show increased *LMNB1* mRNA expression over control (Figure 5.3C), with BR1 having the highest expression overall. However, we do not have brain samples from these patients, and therefore could not test their *LMNB1* expression in white matter.



Figure 5.3: LMNB1 expression in ADLD is higher in white matter than in grey matter or fibroblasts.

(A) *LMNB1* mRNA expression in ADLD duplication patients relative to control in fibroblasts, grey matter, and white matter. ADLD patients have a moderate increase of *LMNB1* in fibroblasts and grey matter, but a much larger expression increase in white matter. K6 and A1 are families with classical ADLD. Fibroblasts were unavailable from A1. n = 3 for control fibroblasts and 1 for ADLD fibroblasts and brain tissues. (B) *LMNB1* mRNA expression in deletion ADLD patients relative to control in fibroblasts and white matter. n = 3 for control fibroblasts and 1 for ADLD fibroblasts and white matter. n = 3 for control fibroblasts and 1 for ADLD fibroblasts and white matter. n = 3 for control fibroblasts relative to control. Fibroblasts and white matter. (C) *LMNB1* mRNA expression in large duplication fibroblasts relative to control. Fibroblasts from K11 family were not available. n = 3 for control fibroblasts and 1 for large duplication fibroblasts.

5.3.4 White matter preferentially expresses duplicated *LMNB1* allele in ADLD

The 3' untranslated region (UTR) of *LMNB1* was PCR amplified and Sanger sequenced to search for heterozygotes of highly polymorphic SNPs, rs1051643 and rs1051644, among ADLD patients and controls. Heterozygotes are necessary to differentiate one copy of *LMNB1* from the other in genomic DNA (gDNA) and mRNA. The 3' UTR was chosen for two reasons: first, the SNPs in UTR regions are more common than in coding regions as they would not result in missense or nonsense mutations and would thus be better tolerated. Second, the SNPs must exist both in the gDNA as well as mRNA, so SNPs within introns would not be appropriate. For these

reasons, the 3' UTR of *LMNB1* was the most likely candidate to find informative SNPs. rs1051643 and rs1051644 were chosen specifically due to their high minor allele frequencies (35% and 45%, respectively) so that we would have a higher chance of identifying heterozygous individuals (Figure 5.4A). Chromatograms from Sanger-sequenced PCR products were analyzed, and the peak heights of heterozygous SNPs within gDNA and mRNA from ADLD samples were compared to controls. cDNA reverse-transcribed from mRNA is used in PCR as a representation of mRNA transcript.

A heterozygous SNP in diploid organisms would normally appear on a chromatogram as two superimposed peaks of different colors indicating two different alleles (Figure 5.4B). As expected, an equal amount of both *LMNB1* alleles are amplified in the gDNA and cDNA from control grey matter, white matter, and fibroblasts (Figure 5.4D and 5.5A), therefore there are no inherent preferences of expressing one copy over another. Heterozygous SNPs in heterozygous duplication patients also present as double peaks, but with the duplicated allele appearing approximately twice as high as the non-duplicated allele (Figure 5.4C). Sequencing of ADLD grey matter and fibroblasts show similar peak heights in gDNA compared to cDNA (Figure 5.4E and 3.5B). White matter samples showed a strikingly different pattern: we were only able to observe the SNP allele corresponding to the duplicated allele in cDNA (Figure 5.4F). This suggests that the duplicated copy of *LMNB1* is preferentially expressed in white matter, but not in other tissues, indicating that the mechanism controlling *LMNB1* might likely be a type of cis-regulatory element.



Figure 5.4: The duplicated copy of LMNB1 is preferentially expressed in ADLD white matter.

(A) Schematic representation of *LMNB1* exon 11 and the 3' untranslated region showing the stop codon, rs1051643 and rs1051644 SNPs, and PCR amplification primers (not to scale). (B) Representation of heterozygous SNP in a nonduplicated locus. Grey bars represent *LMNB1* locus. A representative chromatogram shows two superimposed peaks of equal height. (C) Representation of heterozygous SNP in a duplicated locus. Grey bars represent *LMNB1* locus. A representative chromatogram shows the duplicated allele peak is higher than the non-duplicated allele. (D) SNP sequencing chromatograms in genomic DNA (gDNA, top), grey matter (GM, middle), and white matter (WM, bottom) of control and (E) ADLD brains K6 and CA. (F) WM of ADLD brains show a preferential expression of the duplicated *LMNB1* allele (red arrows).

Fibroblasts from an ADLD patient with a deletion upstream of *LMNB1* have equal amplification of each *LMNB1* allele in gDNA and cDNA (Figure 5.5C). In these patients, there are no copy number variations on *LMNB1* itself, and their fibroblasts do not overexpress lamin B1 compared to controls. Unfortunately, we do not have brain tissues from the same patient, and the

patient from which we do have brain tissue is homozygous across all tested SNPs in the *LMNB1* coding region. Fibroblasts from large duplication patients have similar ratios of duplicated to nonduplicated alleles in gDNA and cDNA (Figure 5.5D), except for one notable exception. Fibroblasts from BR1 preferentially express the duplicated *LMNB1* allele, much like in ADLD white matter.



Figure 5.5: Fibroblasts with LMNB1 duplications express the expected LMNB1 allele ratios.

(A) Heterozygous control fibroblasts show both copies at approximately equal heights in gDNA and cDNA. (B) Fibroblasts from ADLD patients with duplicated *LMNB1* show an approximately 2:1 ratio of duplicated to nonduplicated alleles in both gDNA and cDNA. (C) Fibroblasts from an ADLD patient with a deletion upstream of *LMNB1* show equal expression of both *LMNB1* alleles in gDNA and cDNA. (D) Large duplication families K9 and Port fibroblasts have similar allele ratios in gDNA and cDNA. BR1 shows preferential overexpression of the duplicated allele in fibroblasts (red arrow).

5.4 Discussion

Previous research has linked tandem *LMNB1* duplications to lamin B1 overexpression and ADLD (77, 78, 151). All patients with *LMNB1* duplications, thus far, have been shown to have ADLD. Here, I have shown that a duplication of *LMNB1* is not the only component required for pathogenesis. It was assumed that more copies of the gene led to higher expression. This assumption is correct in certain tissues: for example, we see an increase of lamin B1 expression in fibroblasts from ADLD patients, correlated with the increased copy number. But *LMNB1* may be overexpressed without being duplicated in families with a heterozygous upstream deletion, either from the removal of a TAD boundary that separates *LMNB1* from upstream enhancer elements, or through a different mechanism altogether. Similarly, *LMNB1* duplications themselves do not necessarily lead to ADLD, as families with large duplications that include a significant upstream region have relatively minor autonomic disorders but no ADLD. There must be an alternative mechanism regulating *LMNB1* expression that goes beyond copy number.

My data explain how germline duplications of *LMNB1* or upstream deletions lead to CNSspecific symptoms. The classic tandem duplications involving *LMNB1* as well as upstream deletions that cause ADLD lead to a dramatic overexpression of lamin B1 specifically in white matter, more so than in other tissues, and the duplicated allele is preferentially expressed. Presumably, patients with large tandem duplications and without ADLD do not have greatly increased lamin B1 expression in their white matter, but we do not have brain samples from these families to test because they are unlikely to die from their mild autonomic dysfunction.

These results together hint at a different regulatory pathway controlling *LMNB1* that affects ADLD pathogenesis. I propose the following mechanism: an oligodendrocyte-specific cisregulatory element, likely a silencer, is located upstream of *LMNB1* that regulates its expression.

It must be oligodendrocyte or at least white matter specific due to the fact that we see much higher *LMNB1* overexpression in white matter compared to grey matter and fibroblasts from ADLD patients. It also much be cis-acting because the duplicated *LMNB1* allele is preferentially overexpressed at the transcript level within white matter, overpowering the non-duplicated allele expression. CNVs do not directly affect *LMNB1* expression to a large degree but perturb this silencer instead.

I define a 145 kb critical region, "S Crit," in which this silencer may exist: within the overlap of LD Crit and Del Crit but excluded from all ADLD-causing LMNB1 tandem duplications (chr5:125857895-126003283, Figure 5.6A). In wild-type and large duplication genomes, all copies of LMNB1 have an upstream silencer and are therefore not overexpressed in oligodendrocytes (Figure 5.6B). However, deletions of this silencer region or smaller tandem duplications resulting in an extra copy of LMNB1 without its silencer element lead to the overexpression of lamin B1 specifically in white matter (Figure 5.6C). Furthermore, the insertion of BR1's inverted duplication is between the transcription start of *LMNB1* and the critical region of our putative silencer (81). Therefore, even though BR1 has a large genomic duplication including S Crit, its insertion separates *LMNB1* from its upstream silencer, likely disrupting its suppression of transcription. The cause of BR1's preferential expression of the duplicated allele and much higher lamin B1 overexpression in fibroblasts has yet to be elucidated. This may explain BR1's extremely severe ADLD symptoms and earlier onset. BR1's case also underscores the importance of genetic testing methods that go beyond simple copy number determination. Assays such as aCGH, while effective and capable of high throughput, only give clinicians information on a patient's genomic copy number and not the direction or insertion of a duplication. Determining the size, orientation, and insertion of a genomic duplication through sequencing is crucial for accurate predictions of disease risk.

However, the Sanger sequencing SNP analysis method used here might not be precise enough to draw definitive conclusions on the allelic imbalance (the extent to which one allele is expressed more than the other) of *LMNB1* in ADLD patient white matter. Future projects should use more sensitive allele-specific assays such as droplet digital PCR (152) or single cell RNA-seq (scRNA-Seq) to more accurately determine allele frequencies expressed in mRNA.



Figure 5.6: The critical region of the oligodendrocyte-specific silencer is upstream of LMNB1.

(A) Location of putative oligodendrocyte-specific silencer upstream of *LMNB1*. "Del Crit" and "LD Crit" are the critical regions for ADLD-causing deletions and non-ADLD large duplications, respectively. "ADLD Dup" represents the total span of all known ADLD-causing *LMNB1* duplications. The "S Critical" region represents the area in which the silencer may exist, determined by including all known upstream deletions and duplications and excluding all known ADLD-causing duplications. (B) Mechanism of *LMNB1* silencer "S" preventing *LMNB1* expression and ADLD in wild-type and large duplication oligodendrocytes. (C) Perturbation of the silencer results in *LMNB1* overexpression in white matter, leading to ADLD.

Granted, not all of lamin B1's overexpression is due to a perturbation of this putative silencer. Patients with large and small duplications have increased *LMNB1* transcript in fibroblasts and grey matter compared to normal controls, as well as a corresponding increase of duplicated

allele expression. Copy number clearly still plays a role in the overexpression of lamin B1. However, copy number still does not explain the overexpression of *LMNB1* in ADLD families with upstream deletions, and TAD boundary deletions do not explain the disease outcomes of large versus small genomic duplications of *LMNB1*. More than likely, these disparate outcomes are governed not by multiple regulatory pathways but by a single, common mechanism. As of yet, this model of an oligodendrocyte-specific *cis*-acting silencer offers the most complete and parsimonious mechanism for the genomic regulation of *LMNB1* in white matter, and the consequences of CNVs surrounding it.

6.0 Overall Summary and Future Directions

Over the course of my research, I have investigated the structure and functions of lamin B1 and how it relates to nuclear shape maintenance and onset of ADLD. I aimed to elucidate its 1) structural organization within the nuclear lamina at the INM, 2) role in developing and myelinating oligodendrocytes, and 3) possible regulatory mechanism in white matter. I employed *in vitro* and *in vivo* models of lamin B1 overexpression and deletion, as well as aCGH analysis on patients from families with ADLD.

Much of my research focused on the organization of lamins at the nuclear periphery. While investigating nuclear shape parameters by fluorescently staining cultured MEFs against lamin B1 and lamin A/C, I noticed a peculiar pattern with some of the elongated nuclei: lamin B1 staining was absent in most of their polar ends, resulting in nuclei with uneven lamin B1 staining despite uniform lamin A/C. Initially, I thought this was a staining artifact, but this pattern consistently reoccurred in all subsequent experiments. What began as a questionable immunofluorescence staining evolved into a new paradigm for lamin organization: lamin B1 and lamin A/C form separate meshworks that work together to maintain nuclear structure and integrity. Previous research into the structural organization of the nuclear lamina have shown that A-type and B-type lamins tend to self-organize into independent meshworks (13, 15, 96). However, these efforts lacked the resolution and/or the ability to distinguish protein species to determine how A-type and B-type lamins are organized. We used a form of super-resolution microscopy and saw that the two lamin types have a concentric organization, with lamin B1 localized closer to the periphery due to its farnesylated C-terminus. Form follows function: the properly localized lamin B1 meshwork is responsible for maintaining nuclear shape. It prevents the formation of nuclear blebs arising from the underlying lamin A/C meshwork. Blebs may have a function: they may be useful for rapid morphology changes needed to respond to forces such as sudden contraction of the cytoskeleton. Too much lamin B1 may prevent nuclei from immediately reacting to sudden changes in intracellular forces, as lamin B1 overexpression leads to increased nuclear rigidity (153).

I then sought to expand what we know about lamin B1's function within the cells responsible for myelinating the CNS: oligodendrocytes. Unlike previous research that showed lamin B1 knockouts in neurons leads to severe developmental disorders in mice (11), I found that a lamin B1 deficiency in mature oligodendrocytes does not lead to an obvious phenotype. Despite small changes in myelination and OPC populations early on, cKO mice did not exhibit ambulation deficiencies or reduced life spans. Therefore, lamin B1 might not be necessary for the survival or proper function of mature oligodendrocytes, consistent with previous research linking the rise of miR-23a and concomitant reduction of *Lmnb1* expression to oligodendrocyte differentiation (145, 154). Otherwise, there may be an as-yet unknown compensation for loss of lamin B1.

Previously, miR-23a was found to be upregulated in MS lesions (155) and peripheral blood mononuclear cells (156). It would be interesting to investigate how microRNA levels change in ADLD versus unaffected tissues, such as whether *LMNB1* overexpression leads to a concurrent change of microRNA expression in different tissues, and if changed microRNA levels exacerbate demyelination. Lamin B1 might not just be unnecessary for the proper oligodendrocyte function, it may in fact be detrimental. Elevated lamin B1 levels in oligodendrocytes lead to demyelination, consequently, mature oligodendrocytes may employ a mechanism that suppresses lamin B1 expression.

All in all, structure is function: onset of demyelination may be indirectly influenced by the organization of lamin B1 at the nuclear periphery. While lamin B1 maintains nuclear shape by

preventing the A-type lamin meshwork from protruding outward, it also modulates gene expression through interactions with chromatin. Lamina-associated domains (LADs) are regions of the genome that closely associate with the lamina meshwork at the INM and are associated with tightly condensed heterochromatin and reduced gene expression (157). In particular, lamin B1 associates with LADs that are enriched with repressive histone marks H3K9me3 and H3K27me3 (158), indicating transcriptional inactivation. Meanwhile, chromatin domains within nuclear blebs are enriched with histone marks for active transcription (13). Overexpression of lamin B1, which greatly reduces the formation of blebs and could conceivably increase heterochromatin binding at the nuclear periphery, may therefore lead to aberrant gene expression, specifically the repression of genes that are important for myelin production, such as those involved in lipid synthesis (80), and oligodendrocyte function. While lamin B1 is important for OPC differentiation, myelinating oligodendrocytes cannot tolerate elevated lamin B1 levels, so I propose that they employ a *LMNB1* transcriptional silencer once mature. *LMNB1* overexpression and ADLD onset are due to perturbations of this silencer, and not necessarily from gene duplication.

6.1 Future Directions

6.1.1 Aim 1 future directions

Although we have determined the organization of lamin B1 and A/C at the nuclear periphery, it would be interesting to investigate the organization of individual A-type lamins and lamin B2 as well as their interactions with other nuclear envelope proteins such as nucleoporins

and beta-catenin. This may have important consequences in other molecular pathways such as Wnt signaling in myelination and laminopathies (34, 159, 160).

Previous research has identified lamin B1's effect on cell proliferation and senescence (93, 161), but not the effect of cell proliferation on lamin B1. In non-proliferating retinal neurons, lamin B1 is so long-lived its half-life could be measured in months (135), but during my cell culture experiments, I have seen lamin B1 protein levels decrease significantly after just 3 days in fibroblast culture (data not shown). Overexpression of lamin B1 could theoretically disproportionately affect post-mitotic cells such as terminally-differentiated mature oligodendrocytes in the CNS. This could be tested by administering cell cycle inhibitors to lamin B1-overexpressing fibroblasts in culture and monitor their clearance of protein.

6.1.2 Aim 2 future directions

Even though cKO mice did not exhibit any obvious phenotypes, future work could investigate their ability to remyelinate axons after white matter injury, for example induced through cuprizone (162) or electrolytic lesions.

Considering the *Cnp-Cre* transgene does not express cre at an early enough timepoint to knock out lamin B1 in OPCs, *Olig2-Cre;Lmnb1*^{fl/fl} conditional knockout mice would preferentially be used for future oligodendrocyte-specific lamin B1-knockout models.

6.1.3 Aim 3 future directions

CRISPR-Cas9 knockouts of the critical silencer region that was identified through aCGH studies would provide extremely strong evidence toward the existence of an oligodendrocyte-

specific *LMNB1* regulatory element. This could be done *in vitro* using immortalized oligodendrocyte-lineage cells (163), primary OPCs isolated via immunopanning (164), or in an *in vivo* mouse model. At 145 kb, the critical silencer region is very large, but it can be narrowed greatly via sequential CRISPR knockouts of increasingly narrow regions. Once sufficiently small, regulatory elements could be identified through bioinformatic analysis, and future experiments may be carried out once the nature of the putative *LMNB1* suppressor is more clearly defined. For example, if the element is a transcription factor binding site, individual bases within that site can be mutated, assessing the resulting binding efficiency as well as any changes to *LMNB1* expression. In this case, we could expand ADLD genetic testing to include much smaller mutations such as single base pair changes that are undetectable in aCGH assays.

RNA-seq could be used on ADLD and unaffected control brain samples to evaluate gene expression changes in the white matter compared to grey matter. It would also be a much more sensitive method to determine the allelic skew of *LMNB1* in ADLD patient white matter. However, when using bulk RNA-seq analysis, any differences of gene expression within oligodendrocytes may be masked by the myriad of other cells in the brain, e.g. neurons, astrocytes, microglia, and endothelial cells. Single-cell RNA-seq (scRNA-seq) methods such as CITE-seq (165) would therefore be extremely powerful tools to correlate cell type with *LMNB1* and myelin gene expression within the oligodendrocytes of ADLD patients. Although this may be limited by brain tissue availability and inconsistent harvesting methods from clinicians around the world, data from a very small cohort or even a single patient could provide valuable insight into lamin B1's overexpression within white matter.

Appendix A: Tables of Antibodies

Primary Antibody	Source	Company	Catalog #	WB dilution	IHC/IF dilution
Lamin B1	Rabbit	Abcam	ab16048	1:3000	1:500
Lamin A/C	Mouse	Cell Signaling	4777	1:1000	1:500
Beta-Actin	Mouse	Thermo	MA5-15739	1:5000	
CNP	Mouse	Millipore	NE1020	1:1000	
GAPDH	Mouse	Thermo	MA5-15738	1:5000	
MAG	Rabbit	Invitrogen	34-6200	1:1000	
MOG	Mouse	Santa Cruz	sc-73330	1:500	
PDGFRa	Rat	BD Pharmingen	558774	1:100	
PLP	Mouse	Millipore	MAB388	1:500	
Cleaved Caspase 3	Rabbit	Cell Signaling	9664		1:400
CC1	Mouse	Calbiochem	OP80		1:150
MBP	Rabbit	Abcam	ab40390		1:500
Olig2	Rabbit	Millipore	AB9610		1:150
Olig2	Mouse	Millipore	MABN50		1:200

Table A.1: Table of primary antibodies

Table A.2: Table of secondary antibodies

Secondary Antibody	Source	Company	Catalog #	WB dilution	IHC/IF dilution
Anti-Mouse IRDve					
680LT	Goat	LI-COR Biosciences	926-68020	1:10000	
Anti-Rabbit IRDye					
800CW	Donkey	LI-COR Biosciences	926-32213	1:10000	
		Jackson			
Anti-Mouse Cy3	Donkey	ImmunoResearch	715-165-150		1:350
		Jackson			
Anti-Rabbit FITC	Donkey	ImmunoResearch	711-095-152		1:350
		Jackson			
Anti-Rat Cy3	Donkey	ImmunoResearch	712-165-150		1:350
		Jackson			
Anti-Mouse 6 nm gold	Goat	ImmunoResearch	115-195-068		1:10
		Jackson	111-		
Anti-Rabbit 6 nm gold	Goat	ImmunoResearch	195-144		1:10

Appendix B: Tables of Primers

Genotyping Primers	Sequence (5' - 3')	Product Size (bp)
Cre-	AGGCGTTTTCTGAGCATACCTG	179
Genotyping	AACATTCTCCCACCGTCAGTAC	4/0
Candh	GGCCTGTTTAAGGGAAAGAAGT	324
Gapan	CCCCTTCATTGACCTCAACTAC	
	AACAAACTTGGCCTCACCAG	NU 200
Lmnb1-flox	CTGTGGGACAAAGACCCAGT	Wt = 300 TG = 450
	CCACTTAGCTCGGGGGAGTTT	10 450
TDE I MND1	AGCTCGTTTAGTGAACCGTCAG	600
IKE-LIMINDI	TCAGTGCTGCTTCATATTCTCG	000
	GCGAAGAGTTTGTCCTCAACC	W4 (70
Rosa26-rtTA	AAAGTCGCTCTGAGTTGTTAT	Wt = 650 TG = 340
	GGAGCGGGAGAAATGGATATG	10 540

Table B.1: Genotyping primers

Table B.2: Miscellaneous primers

Misc. Primers	Sequence (5' - 3')	Product Size (bp)
I wuhi I orD	TGAGTTTGACACCAGCCAGAT	Flox = 2100
Lmnd1-LoxP	GGGGTCACATCAAATCACCTA	Wt = 1350, Del = 850
Lmnb1-3'UTR	GGAACCCCAAGAGCATCCAATA	260
	TTATTTACCCTCCCTCCTCAT	509

Real-time PCR Primers	Sequence (5' - 3')
M. Lunchi	GCTGCTCAATTATGCCAAGAAG
NI. LMNDI	GCCGCATCCTTAGAGTTTAGT
M Din	GCCCCTACCAGACATCTAGC
м. <i>Рир</i>	AGTCAGCCGCAAAACAGACT
M Mba	CCCGTGGAGCCGTGATC
м. мор	TCTTCAAACGAAAAGGGA
M Mag	ATGAAGGAGGCTACACCTGC
MI. MOg	CAAGTGCGATGAGAGTCAGC
M Mag	AACCAGTATGGCCAGAGAGC
MI. Mag	GTTCCGGGTTGGATTTTACC
M Cup	GTTCTGAGACCCTCCGAAAA
MI. Chp	CCTTGGGTTCATCTCCAGAA
M Acth	CCACTGCCGCATCCTCTTCC
MI. ACID	CTCGTTGCCAATAGTGATGACCTG
	CATGAAACGCGCTTGGTAGA
	TTGCGCCAGCTTGTACTCATAC
н астр	CACTCTTCCAGCCTTCCTTC
п. АС І В	GTACAGGTCTTTGCGGATGT

Table B.3: Real-time PCR primers

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