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The Expression Pattern and Localization of Linc Complex Components During Mouse Retina Development

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THE EXPRESSION PATTERN AND LOCALIZATION OF LINC COMPLEX COMPONENTS DURING MOUSE RETINAL DEVELOPMENT

By

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Introduction

Schizophrenia is a serious disorder of the brain which impairs higher brain function and has been recently conceptualized as a neurodevelopment disorder (Jaaro-Peled et al., 2009; Deutsch et al., 2010). Recent clinical findings suggest that subtle impairments of cortical lamination may be due to neuronal migration deficits (Deutsch et al., 2010). A large Scottish pedigree led to the identification of the involvement of the DISC1 (*Disrupted-in-Schizophrenia 1*) gene in Schizophrenia (Duan et al., 2007). DISC1 interacts with members of the dynein microtubule motor complex, including LIS1 and NudEL, which are involved in the control of microtubule assembly by the centrosome (Duan et al., 2007). Mutations in LIS1 are linked to lissencephaly, a brain developmental disorder characterized by severe neuronal migration defects (Vallee and Tsai, 2006). Several observations suggest a significant role of Lis1 and other cytoplasmic proteins in connecting microtubules to the nucleus during Neuronal Migration, however the question remains as to how the microtubule network physically connects to the nuclear envelope. Recent evidence suggests that specific proteins of the Nuclear Envelope connect the nucleus to the microtubule network during neurodevelopment.

The Nuclear Envelope

The separation of a cell's genetic material from the surrounding cytoplasm may be the single most important feature that distinguishes eukaryotes from prokaryotes,

which makes the appearance of the nuclear envelope a landmark in biological evolution (Karp, 2002).

The Nuclear Envelope (NE) is a double lipid bi-layer that encloses the genetic information in eukaryotic cells. The Nuclear Envelope serves as a physical barrier to separate ions, solutes, macromolecules and other contents of the nucleus from the cytoplasm. The Nuclear Envelope consists of two parallel membranes, the Inner Nuclear Membrane (INM) and the Outer Nuclear Membrane (ONM). The INM and ONM are separated by the perinuclear space, which is a gap of 30-35nm (Star, 2009). The fusion of the two membranes at annular junctions forms Nuclear Pores that transverse the Nuclear Envelope (Star, 2009). The Nuclear Pores contain Nuclear Pore Complexes (NPC) which can selectively transport macromolecules across the nuclear envelope. The ONM is covered with ribosomes and has multiple connections to the Endoplasmic Reticulum (ER). The ONM shares many of its functions and therefore, the Nuclear Envelope can be regarded as a specialized extension of the ER (Stewart et al., 2007; Starr, 2009).

The Nuclear Lamina is located directly under the INM and adds structural support to the Nuclear Envelope. The Nuclear Lamina provides anchorage sites for chromosomes and the Nuclear Pores and assists in the anchoring of proteins to the INM (Alberts, 2002; Hutchinson, 2002). The Nuclear Lamina is a 10-20nm thick protein meshwork associated with the nuclear face of the INM and is composed of A-and B-type Lamins (Stewart et al., 2007; Razafsky and Hodzic, 2009). A- type and B-type Lamins are members of the type-V intermediate filament family and are believed to be the

ancestors of cytoplasmic intermediate filaments. Lamins are classified as A-type or B-type according to homology in sequence, expression pattern, biochemical properties, and their cellular localization in mitosis (Gruenbaum et al., 2000). Like all Intermediate Filaments, Lamins have a variable globular head domain and are organized around a central domain also know as an α-helical coiled-coil domain. This rod domain consists of four coiled-coil-forming α-helical segments termed 1A, 1B, 2A, and 2B, respectively and each of the four coiled domains contain periodic heptad repeats. The Lamin tail contains a nuclear localization signal and a highly conserved 105 amino acid region that contains an immunoglobulin (Ig) fold (Gruenbaum et al., 2000).

A-type Lamins are expressed in differentiated cells and are developmentally regulated (Gruenbaum et al., 2000; Hutchinson, 2002; Razafsky and Hodzic, 2009). In addition, A-type Lamins have neutral isoelectric points and are completely soluble in the cytoplasm during mitosis (Gruenbaum et al., 2000). B-type Lamins are expressed in every cell and are vital for cell viability (Gruenbaum et al., 2000; Razafsky and Hodzic, 2009). In contrast to A-type Lamins, B-type Lamins have acidic isoelectric points and tend to remain associated with the membranes during mitosis (Gruenbaum et al., 2000). The vertebrate genomes contain one A-Type Lamin gene and two B-Type Lamin genes; B-Type Lamins are termed B_1 and B_2 respectively. There are three genes that encode at least seven different polypeptides of Lamin: Lamins A, AΔ10, C_1 and C_2 are splice variants of the Lamin A gene (LMNA), and Lamin $B_{1\text{-}3}$ (Gruenbaum et al., 2000). Lamin B_3 is a splice variant of the Lamin B_2 gene and Lamin B_3 and C_2 are specific to germ cells and are believed to play a role in chromatin reorganization during meiosis.

In addition to providing structural support to the Nuclear Envelope, the Nuclear Lamina perform many diverse regulatory functions (Gruenbaum et al., 2000; Razafsky and Hodzic, 2009). Current research findings indicate that Lamins regulate chromatin organization and gene expression and influence cell signaling (Wilson and Foisner, 2010). The interactions of the Nuclear Lamina within the INM are mediated by the interactions of the Lamins and INM proteins (Wilson and Foisner, 2010; Burke and Stewart, 2002). Each function requires a multitude of biochemical interactions between Lamins, Chromatin, and regulatory proteins which are responsive to intrinsic and external signals (Wilson and Foisner, 2010). These Lamin binding proteins are located on Lamin, Chromatin and in the nucleoplasm and help anchor NPC and tether chromatin to the Nuclear Envelope, while others regulate signaling, transcription and DNA replication (Wilson and Foisner, 2010, Gruenbaum et al., 2000). In addition, many of the Lamin-binding proteins help to add additional support by reinforcing the nucleoskeleton (Wilson and Foisner, 2010). INM proteins such as lamin-associated peptides 1 and 2 (LAP1, 2), Lamin B receptors, Emerin, and Man1 display decreased lateral diffusion across the INM and have a characteristic nuclear rim-like pattern in immunofluorescense (Razafsky and Hodzic, 2009).

Proetomic analysis of the Nuclear Envelope suggests that the INM is populated with at least 60 different proteins, many of which are uncharacterized (Wilson and Foisner, 2010; Razafsky and Hodzic, 2009). Among those characterized, most bind directly to A-Type or B-Type Lamins or both (Wilson and Foisner, 2010). The up-regulation of some of these proteins during cellular differentiation (Razafsky and Hodzic, 2009) and their involvement in gene expression highlights the importance of fully

characterizing their structure and functions to develop a more intergraded understanding of the Nuclear Envelope.

LINC Complexes

Several proteins located in the inner and outer nuclear membranes have been identified as components of the LINC (Linker of the Nucleoskeleton to the Cytoskeleton) complex (Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010; Crisp et al, 2006). LINC complexes are family of macromolecular assemblies that span the double membrane of the nuclear envelope which create physical connections between the nucleoplasm and the cytoplasm (Razafsky and Hodzic, 2009). In its simplest form, the LINC complex is composed primarily of Lamin-binding, Sun-domain proteins in the INM that interact with ONM-bound KASH proteins in the perinuclear space (Figure 1) (Razafsky and Hodzic, 2009; Starr, 2009; Ostlund et al, 2009; Wilson and Foisner, 2010). KASH proteins also attach to Microtubules, Cytoplasmic Actin, Centrosomes, and Intermediate Filaments which are present in the Cytoplasm. In general, this SUN-KASH interaction works as a Nuclear-Envelope Bridge, which enables membrane proteins to traffic from the ER membrane to either the INM to interact with the lamina, or to the ONM to interact with the cytoskeleton (Starr and Fridolfsson, 2010). This complex has been shown to play a significant role in nuclear positioning and nuclear migration. Disruption of the LINC complexes in the developing Central Nervous System may be associated with neurodevelopmental disorders and neuropathies (Coffinier et al., 2010; Starr and Fridolfsson, 2010; Razafsky and Hodzic, 2009).

SUN Proteins: The Inner Nuclear Membrane Portion of the LINC Complex

Genetic mutations in *C. elegans* embryos were used to develop the current understandings of SUN proteins. Sun proteins were first defined through molecular characterization of the *C.elegans* gene *unc-84* (Starr, 2009). Malone and colleagues discovered that mutations in *unc-84* resulted in defects in nuclear migration and mispositioned nuclei in embryonic hyperderal (hyp 7) and intestinal cells and larval P-cells (Malone et al., 1999; Starr 2009; Razafsky and Hodzic, 2009). In addition, Malone and colleagues discovered that *unc-84* mutations resulted in a disruption of nuclear anchorage in all post-embryonic syncytial cells (Star, 2009). Their observations led to the proposal that the function of *unc-84* and other SUN proteins in general might be to link the mictrotuble-organizing center to the Nuclear Envelope (Raff, 1999). Mutations of the *unc-84* protein results in the loss of centrosome attachment to the nucleus towards the dorsal part of the cell resulting in the nucleus being left behind, ultimately breaking the cell, causing the acentrosomal part of the cell to die (Raff, 1999).

UNC-84 is a transmembrane protein of the Nuclear Envelope which contains an ~ 175-amino acid residue C-terminal that is homologous with the C-termini of *Schizozaccharomyces pombe* protein Sad1 and as well as two mammalian proteins termed SUN1 and SUN2 for '<u>S</u>ad1, <u>*UNC*</u>-84 domain' protein, and this amino acid domain is termed the SUN domain (Star and Fridolfsson, 2010, Razafsky and Hodzic, 2009, Malone et al., 1999). Sad1 was identified as an essential and exclusive protein component of the Spindle pole body (SPB) and normal spindle architecture, and may also play a role in nuclear positioning during interphase (Hagan and Yanagida, 1995; Starr

and Fridolfsson 2010; Malone et al., 1999). Mammalian SUN1 and SUN2 proteins are broadly distributed in mouse tissue and the Sun domain is also found in three other transmembrane mammalian proteins: SUN3, SPAG4 and SPAG4L. SUN3 is predominately expressed in the testes and is mostly localized in the ER (Starr, 2009); SPAG4 is expressed in spermatids and localizes to microtubules of manchette and axoneme (Shao et al., 1999). SPAG4L mRNA has been reported in a wide spectrum of mouse tissues but its localization is uncertain (Razafsky and Hodzic, 2009).

In order for a protein to be classified as a SUN protein, a protein must have three characteristics which include, at least one transmembrane domain and must localize to the INM (Starr, 2009). In addition, the protein must possess a C-terminal domain with homology to other SUN proteins that extend into the perinuclear space of the Endoplasmic Reticulum. Most SUN proteins contain short, coiled-coil regions in their perinuclear domains that aid in dimerization or multerization (Starr and Fridolfsson, 2010).

SUN domain proteins have been evolutionally conserved from yeast to humans and can be found in fungi, plants, animals and protozoan parasites such as Giardia (Starr and Fridolfsson, 2010). The broad evolutionary conservation of SUN domain proteins suggests that they are essential to biological functions (Razafsky and Hodzic, 2009).

SUN protein interactions and KASH protein recruitment

Data suggest that the C-terminal Sun domain of both Sun1 and Sun2 projects into both the perinuclear space and the nucleoplasmic N-terminal region and directly interacts with nuclear lamina components, A-type and B-type Lamins, or Chromatin to regulate gene expression (Starr and Fridolfsson, 2010; Crisp et al., 2006). Membrane proteins that localize specifically to the nuclear envelope do so by a retention mechanism in which they diffuse along the pore membrane domain to the inner nuclear membrane, where they bind to intranuclear components such as Lamins (Lee et al., 2002; Soullam and Worman, 1995). Many SUN proteins interact with Lamins, however different Nuclear Envelope retention mechanisms of mammalian SUN domain proteins may exist. For example, mammalian SUN1 proteins do not require A-type or B-type Lamins for Nuclear Envelope retention however, SUN2 proteins have been mislocalized from the Nuclear Envelope to the Endoplasmic Reticulum in fibroblast lacking, A-type Lamins (Razafsky and Hodzic, 2009). In addition, Lee and colleagues, 2002 demonstrated UNC-84's dependence on Ce-Lamins for stable retention at the Nuclear Envelope. In *C.elegans* embryos lacking Ce-Lamins, UNC-84 proteins drift from the Nuclear Envelop to the Endoplasmic Reticulum (Lee et al., 2002) while Fridkin et al., 2004 reported that *C. elegan* protein SUN-1/MTF-1 remained in the Nuclear Envelope.

SUN proteins play an additional role in the Nuclear Envelop Bridge because they recruit KASH proteins to the ONM. For example, late in *C. elegan* development, UNC-84 is required to recruit KASH proteins ANC-1 and UNC-83 to the Nuclear Envelope (Starr and Fischer, 2005). In addition, missense mutations in the SUN domain of UNC-84 does not disrupt UNC-84 localization to the Nuclear Envelope, but disrupts the

targeting of ANC-1 and UNC-83 to the nuclear envelope (Starr and Fischer, 2005). It is important to note that it is unknown if SUN proteins play a role in recruiting KASH proteins and if it occurs through a direct physical interaction.

Overall, strong evidence supports a model in which SUN proteins form the INM portion of the LINC complex because the SUN proteins anchor the complex to the Nuclear Lamina. Central to this idea, SUN proteins are responsible for the recruitment of the OMN portion of the LINC complex, the KASH proteins (Starr and Fridolfsson, 2010).

KASH Proteins: The Outer Nuclear Membrane Portion of the LINC Complex

The molecular characterization of Klarsicht (*Drosophila*), ANC-1 (*C.elegans*), and Syne/Nesprin-1 and 2 (*mammalian*) proteins lead to the recognition of a family of proteins that conserve C-terminal domains (Starr and Fridolfsson, 2010; Starr and Fischer, 2005; Razafsky and Hodzic, 2009; Starr and Han, 2003). This conserved C-terminal, 60 amino acid residue domain is called the KASH domain (Klarsicht/ANC-1/Syne-1 Homology) (Starr and Han, 2003). KASH proteins are C-tailed, anchored integral transmembrane proteins that localize in the ONM and share a conserved C-terminal domain (Starr and Fischer 2005; Razafsky and Hodzic, 2009). The C-terminal KASH domain serves as a tethering device because it sufficiently targets the protein to the Nuclear Envelope (Starr and Fischer, 2005; Razafsky and Hodzic, 2009). Once present at the Nuclear Envelope, KASH domain proteins require SUN domain proteins

for localization and retention to the ONM (Starr and Fridolfsson, 2010; Wilson and Foisner., 2010; Zhang et al, 2009; Minn et al., 2009; Lei et al., 2009).

The C-terminal KASH domain consists of a single transmembrane region followed by 8-35 amino acids that protrude into the perinuclear space (Zhou and Hanna-Rose, 2010). The largely divergent N-terminal regions of KASH proteins are composed of N-terminal motifs and long, spectrin-like repeats/helical regions, which extend the N-terminus into the cytoplasm where they can interact directly or indirectly with cytoskeletal proteins to perform a variety of functions and regulate diverse cellular processes (Zhou and Hanna-Rose, 2010; Starr and Fischer, 2005).

KASH domain proteins are conserved because they have been identified in several vertebrate and invertebrate proteins (Tzur et al, 2006). To date, there are four known *C. elegan* KASH domain proteins (ANC-1, UNC-83, ZYG-12 and KDP-1), (Wilson et al., 2010; Zhou and Hanna-Rose, 2010), two *D. melanogaster* encoded KASH domain proteins (Klarsicht and MSP 300), (Wilson and Foisner, 2010) and four *mammalian* encoded genes (Nesprin 1-4) (Mellad et al., 2011).

Mammalian KASH proteins are named Nesprins (Nuclear Envelope SPectRIN repeat proteins) (Mellad et al., 2011; Warren et al., 2005). Nesprins display a higher level of complexity in mammals because there are four known genes: Nesprin-1 (SYNE1), Nesprin-2 (SYNE2), Nesprin-3 and Nesprin-4. Nesprin-1 and Nesprin-2 are alternatively transcribed and alternatively spliced to produce more than 20 protein isoforms each, of which vary in size (>1MDa) (Wilson and Foisner, 2010; Warren et al., 2005). The numerous combinations of protein domains displayed by different Nesprin

isoforms allow a wide variety of diversity of function and subcellular localization (Mellad et al., 2011).

All Nesprin proteins contain large central rod domains of variable length containing multiple spectrin repeats which form the backbone of Nesprin proteins (Mellad et al., 2011; Warren et al, 2005). In addition, the central domain contains coiled triple helical bundles which serve to separate the actin-binding domain from the Nuclear Envelope domain and to mediate specific protein-protein interactions (Starr and Han, 2003; Wilson et al., 2010). The C-terminal of Nesprin proteins contains the KASH transmembrane domain which facilitates NE interactions (Warren et al., 2005). Nesprin proteins also contain N-terminal motif domains of actin/plectin binding, Calponin homology domains, which enable Nesprin proteins to interact with different components of the cytoskeleton and orchestrate signaling between the cell membrane and cytoskeleton (Mellad et al., 2011; Warren et al., 2005).

Nesprin-1 (~1MDa in size) and Nesprin-2 (~800kDa) are giant isoforms which contain N-terminal actin binding domains, enabling them to bind to the actin cytoskeleton (Wilson et al., 2010; Warren et al., 2005; Mellad et al., 2011). In addition, the extended central domains of Nesprin-1 and Nesprin-2 contain spectrin-like repeat domains which makes the proteins related to dystrophin, a cytoskeleton protein that connects the actin cytoskeleton to the extracelluar matrix (Starr and Fridolfsson, 2010; Warren et al., 2005; Starr and Han, 2002). Several studies have indicated that Nesprin-1 and-2 work together to regulate the global cytoskeleton and the localization of the mitochondria and other organelles within the cell (Starr and Han, 2002). In a recent study, Nesprin-2 was found

to interact with dynein and kinesin in the neuroepithelium and is highly enriched in photoreceptor nuclei (Zhang et al., 2009; Mellad et al, 2011). Nesprin-3 contains a plectin-binding motif that binds to plakin, a cytoskeletal protein related to plectin that binds cytoplasmic intermediate filaments (Wilhelmsen et al., 2005; Mellad et al., 2011). Nesprin-4 interaction with Kinesin-1 induces nuclear positioning in mammalian cell lines (Roux et al., 2009). Nesprin-4 binds specifically to the Kinesin Light Chain Tericopeptide Repeat domain (KLC TPR) of Kinesin-1, a plus-end-directed microtubule-dependent motor, that may be involved in disolocalizing the Centrosome and Golgi membranes away from the nucleus in secretary epithelial cells (Roux et al., 2009; Wilson and Foisner, 2010). It has been proposed that tissue-specific expression of Nesprin genes may serve to create customized connection between cell types with unique properties (Mellad et al., 2011).

Taken as a whole, current evidence supports a model in which KASH proteins form the ONM portion of the LINC complex and connects the OMN to the cytoskeleton. KASH proteins directly interact with SUN proteins in the perinuclear space forming the Nuclear Envelope Bridge. The largely divergent N-terminal domains interact with a wide variety of proteins of the cytoskeleton to mediate a vast array of cellular processes.

Cellular Functions of LINC Complexes

LINC complexes have been identified as important components in multiple, essential cellular functions such as centrosome association with the nucleus, moving

chromosomes within the nucleus, and nuclear positioning/anchorage, and nuclear migration.

LINC Complexes' involvement in Nucleus-centrosome coupling.

During portions of the cell cycle, centrosomes closely associate with the Nuclear Envelope; LINC complexes mediate this essential interaction (Starr and Fridolfsson, 2010; Mejat and Misteli, 2010). In *C.elegans* embryos, SUN1/MTF-1 recruit ZYG-12 isoforms to the nuclear envelope and mediate the coupling between the nucleus and the centrosome (Malone et al., 2003). In addition, mutations in either ZYG-12 or SUN/MTF-1 result in centrosomes detaching from the nucleus, halting pronuclear migration and leading to lethality of the cell (Starr, 2009; Malone et al., 2003).

LINC complexes are also involved in the tethering of the nucleus to the microtubule-organizing center (MTOC) of yeast. In *S. pombe*, SUN protein, Sad1 and KASH proteins, Kms1 and Kms2 form a physical connection between the spindle pole body (SPB) and centromeric chromatin. This interaction requires INM proteins IMA1 and Nuf2, a component of the Ndc80 centromeric complex (Razafsky and Hodzic, 2009; King et al., 2008). In *S. cerevisiae*, SUN protein Mps3 localizes in a specialized structure of the NE that tethers the SPB to the NE called half bridge (Jaspersen et al., 2006; Razafsky and Hodzic, 2009). The binding of SUN protein Mps3 to Mps2 within the periplasmic space was found to be vital in the formation of the SPB (Jaspersen et al., 2006; Razafsky and Hodzic, 2009). Although Mps2 is not a KASH domain protein, its

C-terminus, like the C-terminal SUN domain of Mps3, was found to be critical for cell viability (Jaspersen et al., 2006). In *Dictoystelium*, SUN1 requires direct binding to chromatin for INM targeting and localization (Xiong et al., 2008). In addition, the binding of SUN1 to chromatin defines the integrity of the nuclear architecture and prevents centrosome hyperamplification and defective spindle formation, which significantly enhances aneuploidy and cell death (Xiong et al., 2008). In mammals, LINC Complex components Emerin and A-type Lamins have been found to play a vital role in the interaction of the centrosome to the NE (Mejat and Misteli, 2010; Razafsky and Hodzic, 2009). Embryonic fibroblasts from Lamin-A knockout mice had an increased distance between nucleus and the centrosome and displayed migration defects and a failure of the centrosome to polarize (Mejat and Misteli, 2010; Razafsky and Hodzic, 2009). Zhang and colleagues demonstrated that Nesprin-1 and Nesprin-2 double knockouts were essential in centrosome-nucleus coupling and Nesprin-1 and Nesprin-2 glia cell knockouts had a drastic increase in distance between the centrosome and the nucleus. In addition, centrosome-nucleus distance in double knockouts of SUN1/2 and Nesprin1/ 2 in glia cells resulted in random distribution patterns indicating that the centrosome was uncoupled for the nucleus (Zhang et al., 2009). Collectively, these results suggest a conserve evolutionary role for LINC complexes in the regulation of the centrosome association with the nucleus.

Moving Chromosomes within the Nucleus and cell division

Recent studies suggest that LINC Complexes are used to transfer forces generated in the cytoplasm to move individual chromosomes within the nucleus (Chikashige et al.,

2006; Starr, 2009). During meiosis, diploid cells are used to generate haploid gametes though a process of two consecutive rounds of chromosome segregation, which is then followed by a round of DNA replication; during this process, homologous chromosomes pair, recombine and synapse. Before homologous chromosomes pair and synapsis begins, meiotic chromosomes are bundled at their telomeres and attach to the INM forming a "chromosomal bouquet arrangement" which aids in meiotic homologous chromosome pairing (Starr, 2009; Harper et al., 2004; Chikashige et al., 2006). Chikashige and colleagues demonstrated that LINC complexes play a vital role in chromosome movement and their results identified SUN protein Sad1, directly interacting with the meiotic-specific bouquet (Bqt) proteins 1 and 2 in fission yeast. More specifically, Btqt1 and Btqt2 connect telomeres to the N-terminus of Sad1 on the nucleoplasmic side of the INM while Sad1 interacts with KASH protein, Kms1 in the perinuclear space (Chikashige et al., 2006). The cytoplasmic domain of Kms1 interacts with dynein light chain (Dlc1), which is a component of the dynein motor complex. Sad1 and Kms1 form a Nuclear Envelope Bridge which connects meiotic telomeres to the dynein motor complex, acting as a driving force for clustering telomeres (Chikashige et al., 2006; Starr, 2009).

In *S. cerevisiae,* the meiotic telomere protein NDj1 mediates telomere attachment to the NE via LINC complexes components. NDj1 specially binds to the N-terminal of SUN domain protein Mps3 and works to stabilize the association of telomeres to the Nuclear Envelope Bridge and assists in the formation of the meiotic bouquet (Conrad et al., 2008). In addition, SUN protein Mps3 has been found to play a critical role in

telomere positioning and anchorage to the NE during mitosis through its N-terminal interaction with Sir4 (silent information regulator protein 4) (Bupp et al., 2007).

In mammals, A-type Lamins and SUN proteins may both play roles in telomere positioning and dynamics (Mejat and Misteli, 2010). The loss of A-type Lamins disrupts nuclear decompartmentalization of telomeres, defects in telomeric heterochromatin, shorting of telomeres and increased genomic instability in mammalian cell lines (Gonzalez-Suarez and Gonzalo, 2009). Current research indicates that the relationship between telomeres and the NE may involve LINC Complex components because SUN1 colocalizes with telomeres between leptotene and diplotene stages of meiosis (Mejat and Misteli, 2010; Razafsky and Hodzic, 2009). Additionally, in $SUN1^{-/-}$ mice, both telomere association with the NE and homologue pairing and synapsis are prevented, resulting in sterility in $Sun1^{-/-}$ mice (Ding et al., 2007).

LINC complex role in nuclear positioning

Proper nuclear positioning and anchorage relative to the cell body is essential for multiple cell processes during mammalian development and has been extensively studied in adult skeletal muscle fibers (Mejat and Misteli, 2010; Lei et al., 2009). Adult muscle fibers are ideal models to study nuclear positioning because mature muscle fibers have multinucleated cells which contain hundreds of evenly distributed nuclei called extrasynaptic nuclei, whereas 3-6 specialized synaptic nuclei aggregate beneath an array of acetylcholine receptors at the neuromuscular junction (NMJ) (Starr and Fridolfsson,

2010; Mejat and Misteli, 2010; Razafski and Hodzic, 2009). In heart and skeletal muscle, Nesprin-1 is abundant in synaptic nuclei and interacts with MuSK (Muscle Specific Kinase) which is an essential organizer of the NMJ (Mejat and Misteli, 2010). Nesprin-1 was identified as having a significant role in clustering nuclei beneath the NMJ. The disruption of the Nesprin-1 gene as well as the overexpression of Nesprin-1 KASH domain in muscle cells resulted in a significant decline in the number of synaptic nuclei clustering beneath the NMJ, suggesting that Nesprin-1 plays a major role in both the recruiting and positioning of the nuclei at the NMJ (Grady et al., 2005; Zhang et al., 2007). Surprisingly, the loss of postsynaptic clustering of nuclei had no effect on mouse muscle function or intervention in mice harboring disruptions in either Nesprin-1 or Nesprin-2. The deletion of both Nesprin-1 and -2 however, resulted in a failure of recruitment of synaptic nuclei to the NMJ, resulting in perinatal death associated with respiratory failure due to diaphragm dysfunction (Zhang et al., 2007).

SUN proteins have been implicated in the role of nuclear positioning of synaptic nuclei in muscle and SUN1 and SUN2 and have been shown to be differentially expressed in muscle nuclei (Mejat et al., 2009). More specially, SUN1 is expressed predominantly in extrasynaptic nuclei and is only marginally detectable in synaptic nuclei, while SUN2 is expressed in all muscle nuclei and is abundant in synaptic nuclei similarly to Nesprin-1 (Mejat et al., 2009). SUN1 and SUN2 have been found to play a significant role in nuclear positioning and anchorage of synaptic nuclei in muscle. SUN1 mutant mice exhibited a modest but significant decrease in the number of synaptic nuclei while Sun2 mutant mice display no phenotype (Lei et al., 2009). The deletion of both SUN1 and SUN2 genes however, results in a drastic loss of synaptic nuclei which results

in the death of mice pups shortly after birth. This suggests that SUN1 and SUN2 play partially redundant roles in nuclear anchorage (Lei et al., 2009). In addition, the lethal phenotype observed in SUN1/SUN2 double knockouts can be rescued by the overexpression of SUN1 in neurons, suggesting that LINC complexes are involved in both the muscle and nerves at the NMJ and are required for the survival of neurons (Lei et al., 2009; Mejat and Misteli, 2010).

Overall, these results indicate that LINC Complexes are essential to connect the Nuclear Lamina to the cytoskeleton, which works to ensure nuclear anchorage of mammalian synaptic and extrasynaptic nuclei.

LINC Complex role in Nuclear Migration

Nuclear Migration is the direct movement of the cell nucleus along a microtubule system which is meditated by motor proteins. Early studies in *C. elagan* proteins UNC-83 and UNC-84 pioneered our current understanding of nuclear migration. Becuase both mutations resulted in nuclear migration deficits, the genetic screening of mutated *C. elegans* Sun protein UNC-84 and C. *elegans* KASH protein UNC-83 suggested an important role of LINC complexes in Nuclear Migration (Wilhelmsen et al., 2006; Malone et al., 1999).

KASH proteins play a major role in connecting the nucleus to molecular motors implicated in nuclear migration. Large isoforms of KASH proteins contain long N-terminal domains which extend away from the nuclear envelope and bind to molecular motors (dynein and kinesin) which interact with microtubule networks to mediate nuclear

migration. Current models suggest that dynein pulls the nucleus toward the centrosome while kinesin pulls nuclei away from the centrosome (Zhang et al., 2009). In *C. elegans*, UNC-83 recruits kinesin-1 to the outer surface of the nucleus where it provides the major force to translocate the nucleus along microtubules in hyp 7 cells (Fridolfsson et al., 2010). In addition, UNC-83 also recruits dynein to the NE though two complexes (BICD-1/EGAL-1/DLC-1 and NUD-2/LIS-1) (Fridolfsson et al., 2010) and these complexes also work to mediate the progression of the migration of the nucleus. Mutation of any complexes or in dynein heavy chain results in migration deficits (Fridolfsson et al., 2010). These results would suggest that UNC-83 is a nuclear specific cargo adaptor for both motors and functions to coordinate bi-directional movements through the interaction with kinesin-1 and dynein (Fridolfsson et al., 2010). In mammals, Nesprin-4 directly interacts with the light chain of kinesin-1; when expressed in HeLa cells, Nesprin-4 recruits kinesin-1 to the NE to facilitate the movement of the nucleus to move away from the centrosome (Roux et al., 2009). These data suggest that Nesprin-4 and kinesin-1 interact to facilitate migration of the nucleus (Roux et al., 2009). In a recent study, both Nesprin-1 and Nesprin-2 redundantly connected the NE to a microtubule network during radial and neuronal migration and interkinetic nuclear migration, while Nesprin-2 was found to interact with dynein/dynactin and kinesin in the neuroepithelium to facilitate nuclear movement (Zhang et al., 2009). Taken together as a whole, these observations suggest an important role of LINC complexes in nuclear migration.

LINC Complexes involvement in Human Disease

Muscle pathologies

LINC complex components are associated with human inherited disease. Among the most noted examples are A-type Lamins (Lamins A and C), encoded by the LMNA gene. Over 200 missense mutations of the LMNA gene are associated with a variety of human diseases called laminopathies or envelopathies (Wilson and Foisner, 2010; Razafsky and Hodzic, 2009). Laminopathies are associated with individual or combined pathologies of bone, tissue, muscle and neurons (Razafsky and Hodzic, 2009). Two fundamental questions have arisen regarding laminopathic proposed pathology: how mutations in the LMNA gene promote disease phenotypes and why certain mutations can give rise to tissue-specific effects. Researchers have proposed two hypotheses to address this discrepancy. The gene-expression hypothesis suggests that mutations of the LMNA gene cause tissue specific changes in gene expression which promotes disease, while the structural hypothesis suggests that mutations of the LMNA gene results in fragile structural integrity of the lamina and subsequent breakage of the nuclear envelope (Hutchinson, 2002). Since NE components and mechanical stimuli trigger transcriptional activation of genes, both the structural and gene-expression hypothesis are likely interrelated (Mejat and Misteli, 2010).

Although laminopathies consist of a large spectrum of affected tissues and diseases, more than 80% of LMNA mutations result in cardiac and/or skeletal muscle pathologies in mammals (Mejat et al., 2009). A vast array of laminopathic mouse models have been developed which recapitulate human disease phenotypes and suggests the involvement of LINC Complexes.

There are two noted LMNA knockout mouse models traditionally used when studying Emery-Dreifuss muscular dystrophy. Mice lacking the *Lmna* gene display normal embryonic development during the first two weeks after birth. By week three, however, *Lmna* knockout pups began to decline in growth and develop cardiac and skeletal myopathies similar to that observed in human Emery-Dreifuss muscular dystrophy and dilated cardiomyopathy (Sullivan et al., 1999; Fatkin et al., 1999). A significant reduction in axon density and the presence of nonmyelinated axons similar to that observed in human peripheral axonopathies also occurs (De Sandre-Giovannoli et al., 2002). In addition, *Lmna* knockout mice share common diseases phenotypes from patients with genetically confirmed Emery-Dreifuss muscular dystrophy. At the cellular level, *Lmna* knockout mice and Emery-Dreifuss muscular dystrophy patients both have clumping heterochromatin, detachment of chromatin from the nuclear membrane, and pseudoinclusions (Mittelbronn et al., 2008). *Lmna* knockout mice die at 6-7 weeks from the progression of cardiac and skeletal myopathies. L*mna* $^{H222P/H222P}$ mice contain a missense mutation identified in a family with autosomal dominant Emery-Dreifuss muscular dystrophy (Arimura et al., 2005). Adult male L*mna*$^{H222P/H222P}$ mice develop reduced locomotion and abnormal stiff walking posture, dystrophic patterns of skeletal muscles, cardiac fibrosis and dilated cardiomyopathy with conduction-system disease; these are reminiscent of the clinical features observed in human Emery-Dreifuss muscular dystrophy patients (Arimura et al., 2005). L*mna*$^{H222P/H222P}$ mice die by 9 months of age due to cardiac arrhythmia.

Both *Lmna*$^{-/-}$ and *Lmna*$^{H222P/H222P}$ knockout mice share common disease phenotypes and molecular defects. Both models exhibited altered epigenetic chromatin

modification and innervations defects as a result of misexpression of electrical activity-dependent genes (Mejat et al., 2008). These molecular defects were observed in both *Lmna* mutant mouse models and in muscle from Emery-Dreifuss muscular dystrophy patients (Mejat et al., 2008). The absence of A-type lamins in *Lmna* knockout mouse models lead to the mislocalization of both SUN2 and Nesprin-1 in synaptic nuclei. The mislocalization of SUN2 and Nesprin-1 proteins results in the structural disorganization of the Neuromuscular Junction (Mejat et al., 2008).

As a whole, these data would suggest that *Lmna* mutations disrupt LINC Complex organizations resulting in the disorganization of NMJ cytoskeleton and muscle innervations. Evidence of LINC Complex involvement in Emery-Dreifuss muscular dystrophy is quite convincing and the disruption of LINC Complexes and related components have been identified in both animal models and human patients. Conflicting evidence exists suggests that the molecular pathogenesis of Emery-Dreifuss muscular dystrophy is not limited to *Lmna* mutations. Clinical studies have indicated that 50% of Emery-Dreifuss muscular dystrophy patients have normal *Lmna* or emerin genes (Warren et al., 2005). Additionally, mutations in Nesprin-1 and Nesprin-2 have been identified in Emery-Dreifuss muscular dystrophy patients (Zhang et al., 2007). In knockout mouse models, disruptions in other LINC complex components have lead to Emery-Dreifuss muscular dystrophy phenotypes. Double knockout mouse models of SUN1/SUN2 and Nesprin 1/2, for example, display NMJ defects comparable to those observed in LMNA knockouts (Mejat et al., 2009; Zhang et al., 2007). Mutations in LINC complex components have been indicated in non-muscle diseases, such as autosomal recessive cerebellar ataxia (Gros-Louis et al., 2007). Adult-onset autosomal dominant

leukodystrophy (Lin et al., 2011), Charcot-Marie-Tooth axonal neuropathy (De Sandre-Giovannoli et al., 2002) may play a significant role in the development of Lissencephaly, a neuronal migration disease (Wilson, 2010; Morris et al., 1998).

Neuronal diseases

Recent evidence indicates that LINC complex components are associated with neuronal diseases such as Charcot-Marie-Tooth axonal neuropathy, adult-onset autosomal dominant leukodystrophy and autosomal recessive cerebellar ataxia.

Charcot-Marie-Tooth axonal neuropathy is an inherited autosomal dominant disorder which results in sensorimotor peripheral neuropathy affecting 10-40/100,000 individuals. Like other peripheral neuropathies, symptoms of the condition include muscle weakness and atrophy, loss of reflexes, and loss of sensation in the distal parts of the limbs and these symptoms appear in childhood or adolescence and progress slowly (De Sandre-Giovannoli et al., 2002; Kandel, 2000). Charcot-Marie-Tooth disorder Type 2 (CMT2) is characterized by a slight to absent reduction of nerve-conduction velocities accompanied by a loss of large myelinated fibers and axonal degeneration (De Sandre-Giovannoli et al., 2002). Genetic mapping of patients with CMT2 indicated that missense mutation in LMNA gene results in an arginine to cysteine change at position 298(R298C) in the lamin alpha-helical rod domain and thus affects the production of all four isoforms produced by the LMNA gene (A, AΔ10, C_1 and C_2) (De Sandre-Giovannoli et al., 2002). Ultrastructural exploration of sciatic nerves of Lamin-A knockout mice revealed a strong reduction of axon enlargement and the presence of nonmyelinated axons, all of which were highly similar to the phonotypes of human peripheral

axonopathies (De Sandre-Giovannoli et al., 2002). Taken as a whole, these results suggest a fundamental function of Lamin A/C during the development of the peripheral nervous system.

Adult-onset autosomal dominant leukodystrophy (ADLD) is a slow progressing neurological disorder which features wide spread myelin loss in the central nervous system (Padiath et al., 2006). ADLD is distinct from other demyelinating disorders in that in the presence of myelin loss, oligodendroglia are preserved and both astrogliosis and neuronal damage is absent (Lin et al., 2011; Padiath et al., 2006). Neuropathological observations indicate that ADLD patients have most significant white matter loss in the brain extending from the frontoparietal region to the cerebellum (Lin et al., 2011). Genomic analysis of ADLD patients indicate that ADLD was caused by the duplication of the LMNB1 gene, resulting in increased gene dosage in brain tissue (Padiath et al., 2006). The overexpression of LMB1 gene in *Drosophila melanogaster* and mammalian cell lines resulted in degenerative phenotypes and abnormal nuclear morphology (Padiath et al., 2006). Efforts were made to recapitulate the ADLD phenotype in primary culture systems by using lentivirus to induce moderate overexpression of LMNB1 in oligodendrocytes. This overexpression leads to drastic reductions of myelin protein expression and defects of differentiation, which would suggest that the genes required for myelin proteins and oligodendrocyte maturation are sensitive to changes in nuclear envelope (Lin et al., 2011). Taken as a whole, these results suggest that proper expression of Lamin B1 proteins is essential to the generation and maintenance of myelin within the brain and the central nervous system.

Autosomal recessive cerebellar ataxia (ARCA) is a neurological disorder characterized by cerebellar atrophy accompanied by impaired walking with a lack of coordination of gait and limbs. Many affected individuals have additional neurological symptoms such as pyramidal features, peripheral neuropathy, extrapyramidal signs, cognitive loss or retinopathy (Gros-Louis et al., 2007). Genome-wide linkage analysis of patients with ARCA indicated that mutations in *SYNE-1* genes which encode multiple Nesprin-1 protein isoforms, cause autosomal recessive cerebellar ataxia (Gros-Louis et al., 2007). Immunohistochemistry and *in siti* hybridization data of normal mouse brain cross sections indicated strong expression of Nesprin-1 in Purkinje cells of the cerebellum and neurons of the olivary region of the brain stem (Gros-Louis et al., 2007). Since Nesprin-1 is highly expressed in precisely positioned Purkinje cell nuclei in the cerebellum, one may infer that loss of the Nesprin-1 may lead to deficits in cerebellar architecture leading to ARCA phenotype (Gros-Louis et al., 2007).

Microtubule Network Interaction with Interkinetic Nuclear Migration and Neuronal migration

The formation of the Central Nervous System consists of several complex processes that must be carefully orchestrated during development. Neuronal progenitors expand though proliferation, differentiate and exit the cell cycle to generate a plethora of neural and glial cell types found in the CNS. The newly postmitotic daughter cells migrate to their specified locations to form precise synaptic connections (Donovan and Dyer, 2005). The two major cellular processes involved in this formation are Interkinetic Nuclear Migration and Neuronal Migration.

Interkinetic Nuclear Migration is the process in which the nucleus migrates within the cytoplasm of elongated neuroepithelial progenitor cells (Baye and Link, 2008). During Interkinetic Nuclear Migration the centrosome (the microtubule organizing center) is maintained along the apical surface while the nuclei of the progenitor cells oscillate from the apical to basal surfaces (or central to peripheral) of the neuroblastic layer (Figure 2). In addition, Interkinetic Nuclear Migration occurs while coordinating the progression of the cell cycle; nuclei at the M phase are positioned at the apical surface while nuclei in the G1-phase, S-phase, and G2-phases are located at a more a basal position. Cells in G1 phase are able to respond to localized intrinsic and/or extrinsic signals to promote cell fate commitment and exit the cell cycle (Baye and Link, 2008). The function of Interkinetic Nuclear Migration is not certain, however recent observations suggest that the preservation of apical and basal processes permits the maintenance of localized protein signaling complexes throughout the cell cycle and modulates cell fate differences between neuroepithelial cells (Baye and Link, 2007). Once neuronal precursors exit the cell cycle, several of them will undergo neuronal migration.

Neuronal Migration is a process by which neurons travel from their place of origin to their final position within the CNS. During Neuronal Migration, neurons that differentiate together during Interkinetic Nuclear Migration migrate together to their final positioning and is the fundamental process for the development of laminary structures in the mammalian brain, which includes the cortex, hippocampus, midbrain and hindbrain (Coffinier et al., 2010; Zhang et al., 2009). The final position of a neuron is a strong determinant for its maturation and the establishment of neuronal connections. The

consequences of defective neuronal migration are quite severe, and are illustrated in the disease pathology of lissencephaly (Coffinier et al., 2010).

Lissencephaly is a brain developmental disorder characterized by severe neuronal migration defects, smooth brain surface, mental retardation and seizures. This disease is classified into two categories: Type1 and Type 2. Type 1 Lissencephaly is caused by mutations in the *LIS*1 gene, while Type 2 Lissencephaly is caused by mutations in the *DCX* gene which encodes the protein doublecortin (Vallee and Tsai, 2006). Lissencephaly has received a considerable amount of attention in the field of cell biology and neuroscience because the disease has a striking feature: differentiated neurons are produced however, the majority of them are mislocalized, resulting in abnormal cortical layering.

Both doublecortin and LIS1 proteins interact with microtubules. LIS1 is part of a complex which consists of NDEL1, 14-3-3 episilon and other cytoplasmic proteins which are essential for the regulation and localization of cytoplasmic dynein and microtubule dynamics (Wynshaw-Boris, 2007; Pramparo et al., 2010). The interaction of LIS1 and cytoplasmic proteins are critical for Interkinetic Nuclear Migration and Neuronal Migration. Doublecortin proteins are microtubule-binding proteins that aid in the stabilization and binding of microtubules in migrating neurons (Gotz et al., 2005; Faulkner et al., 2000; Gleeson et al., 1999). Recent observations suggest that LIS1 and doublecortin proteins interact in tandem or indirectly via LIS1 complex proteins to interact with microtubules to facilitate Interkinetic Nuclear Migration and Neuronal Migration (Tanaka et al., 2004; Pramparo et al., 2010).

Mouse models have aided in the understanding of LIS1 and DCX function in brain development and have resembled disease phenotypes of human Lissencephaly. Mice with reduced levels of LIS1 exhibited disorganized cortical layers of the hippocampus, cerebellum and olfactory bulb due to neuronal migration defects (Hirotsune et al., 1998). These observations were further supported by developmental histological analysis, BrdU birthdating experiments and *in vitro* migration experiments of cerebellar granule cells (Wynshaw-Boris, 2007). In addition, mice with reduced LIS1 levels displayed abnormal neuronal migration and defects in Interkinetic Nuclear Migration of neuroepithelial cells (Gambello et al., 2008). Both Dcx mutant mice and Dcx RNAi knockdowns display neuronal migration defects. Dcx mutant males displayed significant cortical disorganization and a depletion of progenitor pools, while Dcx RNAi knockdowns displayed mispositioned neurons in laminar position as a result of delayed migration (Bai et al., 2003; Pramparo et al., 2010). Taken as a whole, these observations suggest a critical role of Lis1 and other cytoplasmic proteins in connecting microtubules to the nucleus during Interkinetic Nuclear Migration and Neuronal Migration, however the question remains as to how the microtubule network physically connects to the Nuclear Envelope.

LINC Complex connect microtubule networks to the Nuclear Envelope during Interkinetic Nuclear Migration and Neuronal Migration

Two recent studies have significantly advanced our understanding of the role of the LINC Complex in mammalian Central Nervous System. These studies identified a role for SUN, Nesprin and Lamin B2 proteins in Nuclear Migration and Neuronal

Migration. SUN (1/2), Nesprin (1/2) double knockouts and Lamin B2 knockout mice displayed reduced brain size with lamination defects of the cerebral cortex, hippocampus, cerebellum (Zhang et al., 2009; Coffinier et al., 2010). Both SUN (1/2) and Nesprin (1/2) double knockouts share common features such as severe lamination defects of the cerebral cortex, hippocampus, cerebellum and olfactory bulb which suggests a redundant role of SUN1 and 2 in brain development (Zhang et al., 2009). In addition, SUN (1/2) and Nesprin (1/2) double knockouts display enlarged ventricles which mimics a common phenotype found in Schizophrenia patients (Zhang et al., 2009; Jaaro-Peled et al., 2011). Mice deficient in SUN (1/2), Nesprin (1/2), and Lamin B2 displayed abnormal brain morphology by E13.5 and pronounced abnormalities late in embryonic development which remained perinatally (Zhang et al., 2009; Coffinier et al., 2010). BrdU birthdating labeling experiments indicated abnormal cortical layering and inverted patterning in SUN(1/2), Nesprin (1/2) double knockouts and Lamin B2 knockout mouse brains, suggesting that cells were defective in their ability to migrate into more superficial layers of the cortex (Zhang et al., 2009; Coffinier et al., 2010).

LINC Complex proteins SUN 1/2 and Nesprin 1/2 play dynamic roles during brain development. Nesprin (1/2) double knockouts displayed identical lamination defects observed in SUN1 and 2 knockouts. Nesprin-2 mutants displayed defective laminary structures of the cerebral cortex and hippocampus however, Nesprin-1 mutants showed no obvious effects (Zhang et al., 2009). This would suggest that both Nesprin-1 and Nesprin-2 have redundant roles in the development of the hindbrain and midbrain, while Nesprin-2 is essential for the laminary structure formation in both the cerebral cortex and hippocampus (Zhang et al., 2009).

In the embryonic cortex, SUN1 and SUN2 are localized at the NE and are required for the anchorage of Nesprin-2 at the NE (Zhang et al., 2009). In addition, SUN1, SUN2 and Nesprin-2 colocalized with Lamin B at the Nuclear Envelope while Nesprin-1 displayed an association to LIS1 (Zhang et al., 2009). Time lapse recordings of EGFP of labeled centrosomes and nuclei demonstrated that although the centrosomes migrated forward for long distances, nuclei of Sun (1/2) double knockouts and Nesprin-2 knockouts fail to migrate, suggesting that SUN1, SUN2 and Nesprin-2 play an essential role in Interkinetic Nuclear Migration and Neuronal Migration. More specifically, SUN1, SUN2 and Nesprin-2 are essential for the coupling of the nucleus to the centrosome during radial migration (Zhang et al., 2009). Immunoprecipitation experiments indicated that both Nesprin-1 and 2 interacts with the cytoplasmic dynein/dynactin complex during brain development (Zhang et al., 2009).

Taken as a whole, these observations suggest that SUN proteins 1/2 connect to Lamin B (or other nuclear lamins) in the Nucleoplasm and Nesprin (1/2) in the cytoplasm to form a LINC complex in young neurons. In addition, this complex connects the nucleus to the centrosome to transfer forces of microtubule motors proteins, cytoplasmic dynein, or kinesin to facilitate controlled movement of the nucleus during Interkinetic Nuclear Migration and Neuronal Migration.

These observations provide compelling evidence establishing a significant role of LINC Complex components involvement in Interkinetic Nuclear Migration and Neuronal Migration during Neurodevelopment. However, further investigation is essential for the

establishment of SUN/KASH interaction in the manifestation of diseases such Lissencephaly and Schizophrenia.

To further complicate the matter, the human brain consists of more than 100 billion neurons, which form intergraded networks and require precise choreography during development. A simplistic model is necessary to establish a true relationship and understanding of LINC Complex contributions in mammalian neurodevelopment.

The functional role of LINC complex components in Interkinetic Nuclear Migration and Nuclear Migration/anchorage is well established however, little is known about the temporal expression and localization of LINC Complexes during CNS development.

Mammalian retinal development provides an excellent model to analyze the expression pattern of LINC Complexes in the CNS. The retina is a uniquely laminated structure and is comprised of three distinct cell body layers: the outer nuclear layer (ONL), inner nuclear layer (INL) and the retinal ganglion cell layer (RGC), separated by the outer (OPL) and inner plexiform layer. Within these three cell body layers are six cell types: photoreceptors in the ONL, bipolar cells, amacrine and horizontal cells in the INL, ganglion cells in both the RGC and glia and Muller cells located in all three layers. Much like other laminary structures of the mammalian brain, the formation of the mammalian retina consists of Interkinetic Nuclear Migration within retinal precursor cells and Neuronal Migration of post-mitotic neurons during laminar positioning.

In this study, the expression pattern of A-type and B-type lamins, SUN and Nesprin proteins were characterized during mouse retina development via RT-PCR and Immunofluorescence microscopy.

Methods and Materials

Animals

All experimental procedures were performed on wild type C57BL/6 mice obtained from Jackson Laboratory (Bar Harbor, ME) and housed in our animal facility. All mouse breeding and experimental procedures were carried out in accordance to the general guidelines published by The Association for Assessment and Accreditation of Laboratory Animal Care. All animal related procedures were approved by the Washington University Animal Studies Committee. Mice were sacrificed at varying time points, from embryonic day 11.5 (E11.5) throughout adulthood. The day of conception was designated as E0.5, and the day of birth was designated as postnatal day 0 (P0).

Reverse Transcription and Polymerase Chain Reaction Analysis of LINC Complex Transcripts

Expression of LINC Complex components were evaluated by reverse transcription and polymerase chain reaction (RT-PCR) analysis. Mouse retinas were extracted at E11.5, E18.5, P2, P25 and P75. Retina RNA was isolated with Trizol reagent (Invitrogen). Total RNA quality was visually analyzed by agarose gel electrophoresis and by the A260/A280 ratio of optical density measured by the UV/ Visible Spectrophotometer (Bio Wavell). RNA (1µg) was reverse transcribed into cDNA using Super Script II Reverse Transcriptase (Invitrogen). The RT-PCR was carried out for 30 cycles with an initial denaturing temperature of 94°C for 1 minute, a denaturing temperature of 94°C for 30 seconds, an annealing temperature of 60°C for 1 minute and final extension temperature of 68°C for 2 minutes. A listing of sense and antisense

primers and product size are listed in table 1. RT-PCR products were run on 2% agarose gel and were imaged in non saturating conditions. Each time point was quantified relative to the GAPDH signal (set at 100%) using Gene Tool software (G box, Syngene).

Western Blot

To assess LINC Complex proteins in mouse retina, E14.5 and P75 retinas and C2C12 mouse myoblast cells were homogenized with glass beads (Bullet Blender) in Laemli buffer with Complete^{TM} protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany). The lysate was centrifuged at 10,000 rpm for 10 minutes. The supernatant was then collected and protein concentration was determined via BCA Protein Concentration Assay (Thermo Fischer Scientific, Inc). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 1X Tris buffered saline with Tween-20 (TBST), 5% milk for one hour at room temperature. Primary antibodies goat anti Lamin B1 (1:100; Santa Cruz, SC-6216), goat anti Lamin A/C (1:100; Santa Cruz SC-6215) and Nesprin 3 (1:100; Hodzic lab) were blotted in 1X TBST, 5% milk overnight at 4°C. Secondary antibody HRP conjugates were blotted in 1X TBST, 5% milk for 1 hour at room temperature and the signals were detected with Super Signal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific, Inc).

Histological preparation and immunofluorescent staining

Immediately following sacrification, mouse eyes were dissected and rinsed in phosphate-buffered saline (PBS) solution and fixed in 4% paraformaldehyde/PBS solution for 30 minutes. Mouse eyes were then rinsed in PBS solution, and mouse retinas were dissected and embedded in 4.5% low melt agarose/PBS and incubated at 60°C for 30 minutes. Embedded retinas were allowed to cool at 4°C and embedded retinas were sectioned (60 µm) with a vibratome. Blocking was performed by incubating the sections at room temperature in PBST (0.1% Trition X-100 in PBS) with 5% donkey or goat Serum for 30 minutes. Sections were subsequently washed three times in PBST for five minutes and incubated overnight with indicated primary antibodies diluted in PBST with 5% donkey or goat serum at 4°C. Following the overnight incubation, sections were washed three times in PBST for five minutes and incubated with Alexa conjugated secondary antibodies (1:2000; Invitrogen,) for two hours. For DNA visualization, retina sections were washed three times in PBST for 30 minutes and stained for 10 minutes with DAPI (4,6-diamidino-2-phenylindole)(1:20,000; Sigma, D8417) and mounted on a glass microscope slide with fluorescent mounting media (DAKO). The antibodies used in this study were goat anti Lamin A/C (1:50; Santa Cruz, SC-6215), goat anti Lamin B1 (1:50; Santa Cruz, SC-6216), mouse anti Lamin B2 (1:50; Invitrogen), mouse anti Syntaxin 1 (1:50; Synaptic Systems, 110 011) and rabbit anti PKCα (1:400; Santa Cruz, SC-208). Images of retina sections were captured on a fluorescence microscope (TE Eclipse, Nikon) and images were manipulated with NIS-Element imaging software (Nikon).

Results

Expression patterns of LINC complex transcripts in mouse retina

The purpose of this experiment was to evaluate expression patterns of LINC Complex transcripts in mouse retinas. RNA was isolated from embryonic postnatal and adult mouse retinas and RNA was reverse transcribed to make cDNA which was used as a template for RT-PCR. RT-PCR results indicated that Emerin, Lamin B1, and Nesprin-2 are expressed in relatively high abundance early in embryonic development and Emerin expression levels remain constant throughout development (Figure 3). Lamin B1 expression levels increase throughout early embryonic development and peaks at E18.5. Nesprin 2 expression levels remain stable from early embryonic development through early postnatal periods until it suddenly spikes by P25 and remains above the basal level throughout adulthood. SUN1 expression levels in development are far more pronounced than SUN2. In addition, SUN1 transcripts are alternatively spliced in a developmental specific manner. Cloning and sequencing of Sun1 splice variants revealed the alternative splicing of axon 7, 8, and 9 which encodes 123 amino acids from the central domain of SUN1 nucleoplasmic region (Figure 4). Early in development, the full length fragment and the 4 splice variants are present in the embryonic retina. By P75, the full length and delta9 isoforms are absent, while the delta 7, delta 7, 9, and delta 7, 8, and 9 isoforms remain present in adulthood. Nesprin 3 is absent in embryonic and postnatal development; by P25 Nesprin 3 dramatically appears and remains present throughout adulthood.

Immunolocolization of LINC Complex components in retina

Experiments were conducted to identify the presence and expression levels of LINC Complex components in mouse retina. Immunolocalization of LINC complex components was identified in embryonic and postnatal retinas and adult retinas. Early detection of LINC Complex components in P0 mouse retinas revealed low expression levels of both Lamin A/C and SUN1 in retinal precursor cells in comparison to Lamin B1 and LAP2 (Figure 5). Lamin B1 is expressed earlier in embryonic stages and remains prevalent during postnatal development. Lamin B2 displays dynamic expression patterns (Figure 6). In early development Lamin B2 is dormant until P12 and has a strong signal by P18. Lamin B1 is expressed throughout the retina and is evenly distributed through the three cell layers. In contrast, Lamin B2 is expressed only in the INL and RGC layers from P12 through adulthood. Lamin A/C displays a preferential expression in the RGC and amacrine cells within the INL (Figure 7). SUN1 is also abundant in the RGC and is uniformly distributed throughout the INL. Both Lamin A/C and SUN1 are weakly expressed in rods but display prominent signals in cones.

Western Blot Analysis

Western blot analysis was conducted to confirm the findings of the transcript analysis on a protein level. E14.5 and P75 retina lysates were blotted against LINC Complex antibodies. Western blot analysis revealed consistent expression of Lamin B1 in E14.5 and in P75 retina lysates and an absence of Nesprin 3 in E14.5 retina lysates which confirms the findings of the transcript analysis (Figure 8).

Discussion

This investigation was conducted to evaluate expression patterns and localization of LINC Complex components in developing mouse retina. The functional roles of LINC Complex components in nuclear migration and anchorage has been established, however the temporal expression and location of LINC Complex components during neural development was unknown. Using RT-PCR and immunofluorescence microscopy and a western blot, we characterized the expression pattern of A-and B-type lamins, SUN proteins and Nesprins during mouse retinal development and showed that the expression patterns of SUN1, Lamin B2 and Nesprin-3 are developmentally-regulated and we showed that Lamin A/C and Lamin B2 are preferentially associated to a restricted set of neurons. This was the first study to classify LINC Complex expression patterns in neural tissue. This is also the first study to indicate SUN1 isoforms and the dynamic expression pattern of Lamin B2 within neurons. Several studies have indicated redundant roles of A-type and B-type lamins in cell development. This study suggests that type-A and B lamins play significant but perhaps unrelated roles in neuroepithelial cell development. The late expression pattern of Nesprin-3 suggests that it plays no role in Neurogenesis, however it may play an important role in the further establishment of laminary positions in late stages of neural development. Zhang et al., 2009 established a relationship between LINC complex components to that of motor and adaptor proteins involved in Interkinetic Nuclear Migration and post-mitotic neuronal migration. The discovery of expression patterns of LINC Complex components during Neurogenesis may be an important area of research which requires further inquiries. Future studies should address the significance of LINC Complex component expression patterns and their relationships

to Interkinetic Nuclear Migration and post-mitotic Neuronal Migration. This information may be valuable for the understanding of LINC Complex involvement in neuronal diseases such as lissencephaly and schizophrenia.

Table 1. Primer pairs used for RT-PCR experiments

Gene	Sense	Antisense	Amplicon (bp)
SUN 1	CCGCAGCTTGCGTCTGGTCA	CAGCCAGCCAGAGCACCGAC	959
SUN 2	CGAAGCCCGAGTCCAGCAGC	CGTGCACCCGGAAGCGGTAG	986
Emerin	AGCCGCCGTGGACTCAGACA	GGCCCCAGAGTGGGACCTGT	485
Lamin A/C	CAATGACCGCCTGGCCGTGT	GCATCCTCGCCCGCATCTCC	944
Lamin B1	GAGCGCGCCAAGCTCCAGAT	GCTCGCCTCCGACTCCTCCA	987
Nesprin 1	CAACGGCGCCCCACCTTGAA	CGGCAGATGCTGACGCTTCCA	535
Nesprin 2	GGCCGAGCCTCCCTCTGACA	GGTGGCCCGTTGGTGTACCG	924
Nesprin 3	CCCTGCTCCAAGCACTGGGC	TCCACCGCTTCTGCCTCCGA	633
GAPDH	CCACCTCTGTGGACCAGAAT	CATTTTGCACCCCTTCCTAA	670

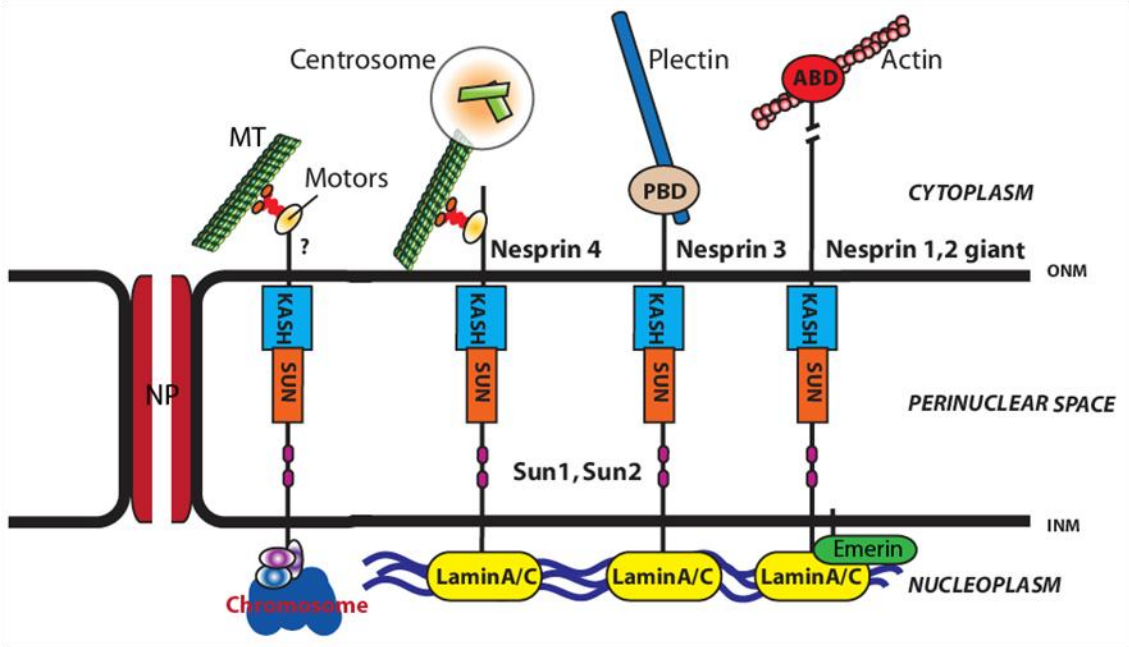


Figure 1. LINC Complex Topology and Function.

In the perinuclear space, SUN and KASH domain proteins physically connect to the nuclear lamina and to cytoskeletal elements such as actin and microtubule network. This connection facilitates both nuclear migration and anchorage at specific locations within cells and syncytia and may mediate chromosome dynamics and Nucleus-centrosome coupling. MT: Microtubules; PDB: Plectin-binding domain; ABD: Actin-Binding domain; INM, ONM: Inner and Outer Nuclear Membranes; NP: Nuclear pore.

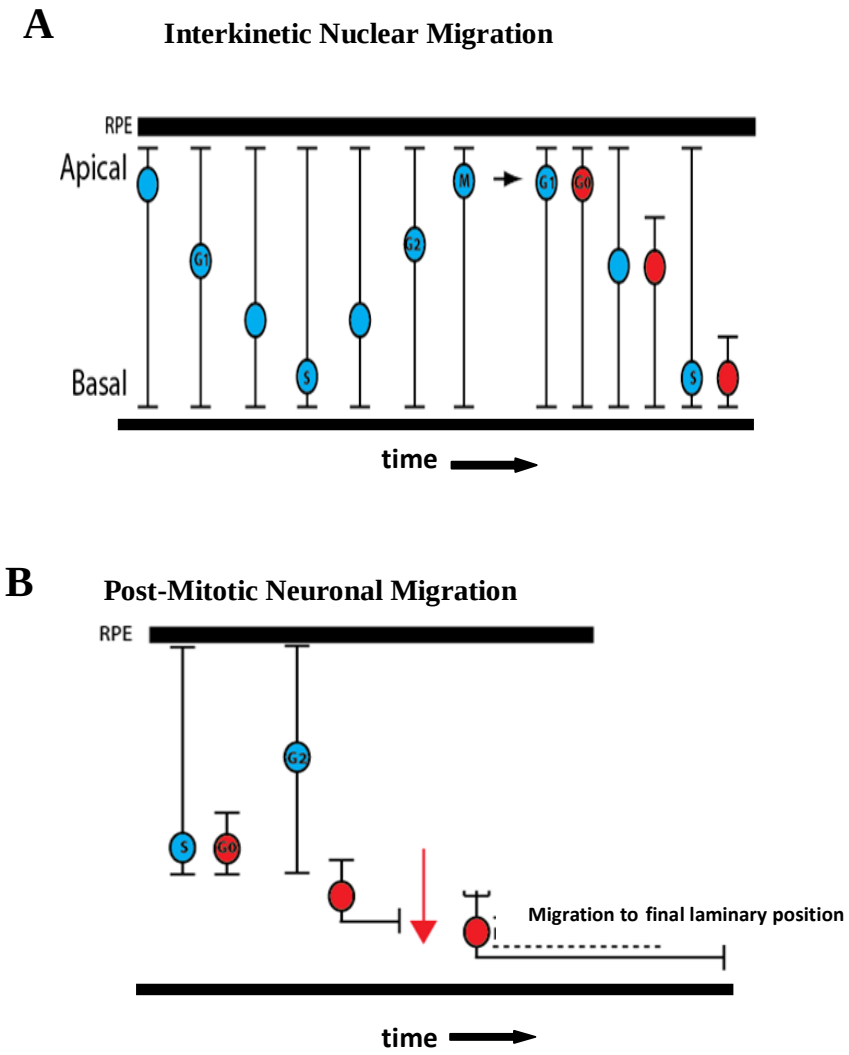


Figure 2. Interkinetic and Post-Mitotic Neuronal Migration during Neurogenesis.

(**A**) During Interkinetic Nuclear Migration, nuclei of neuroepithelial cells migrate within the cytoplasm of eligated neuroepithelial progenitor cells. Progenitor cells migrate from apical to basal surfaces. Nuclei at M-phase are positioned at the apical surface while nuclei in G1-S1 and G2 phases are located at a more basal position. (**B**) Once neuronal precursors exit the cell cycle, many under go Neuronal Migration. Neurons that differentiate together during interkinetic nuclear migration migrate together to their final laminar position.

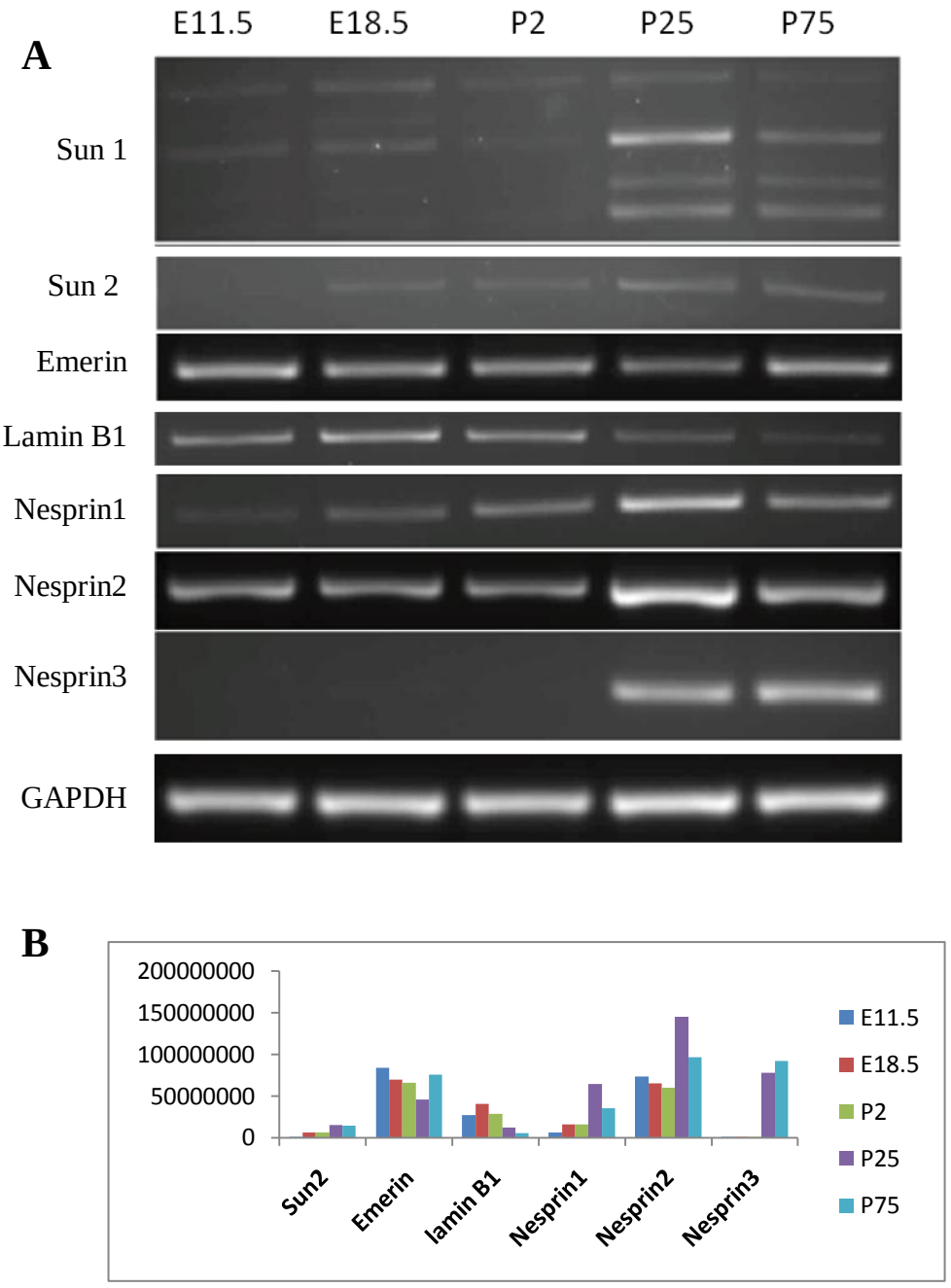
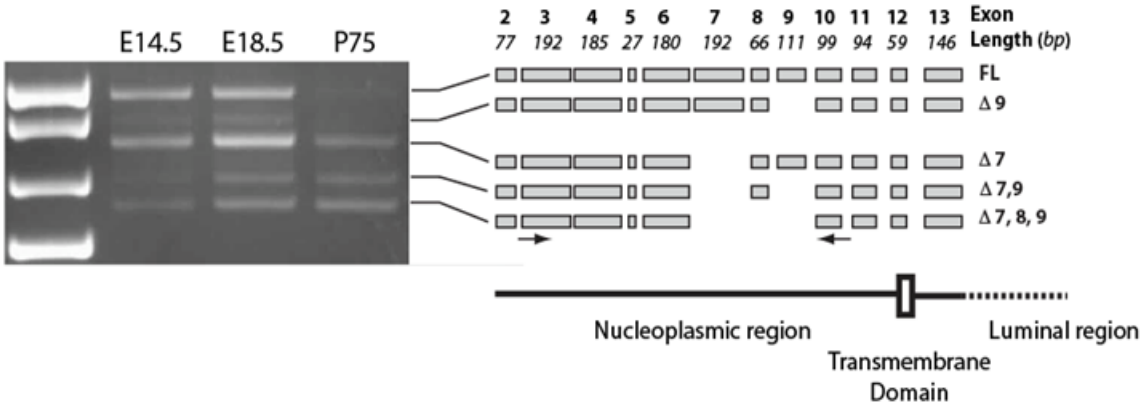


Figure 3. Temporal Expression of LINC Complex Transcripts.

(A) One microgram of total RNA extracted from whole retinas (E11.5 to P75) was reverse- transcribed and PCR-amplified using primers of similar melting temperature (60C). PCR was carried out for 35 cycles with 1 minute elongation. Gels were imaged in non-saturating conditions and the signals normalized to GAPDH using the GeneTools software (Gbox, Syngene). **(B)** The relative abundance of SUN2, Emerin, Lamin B1, Nesprin-1, Nesprin-2, and Nesprin-3 transcripts (arbitarty units) were quantified for each time point.

A



B

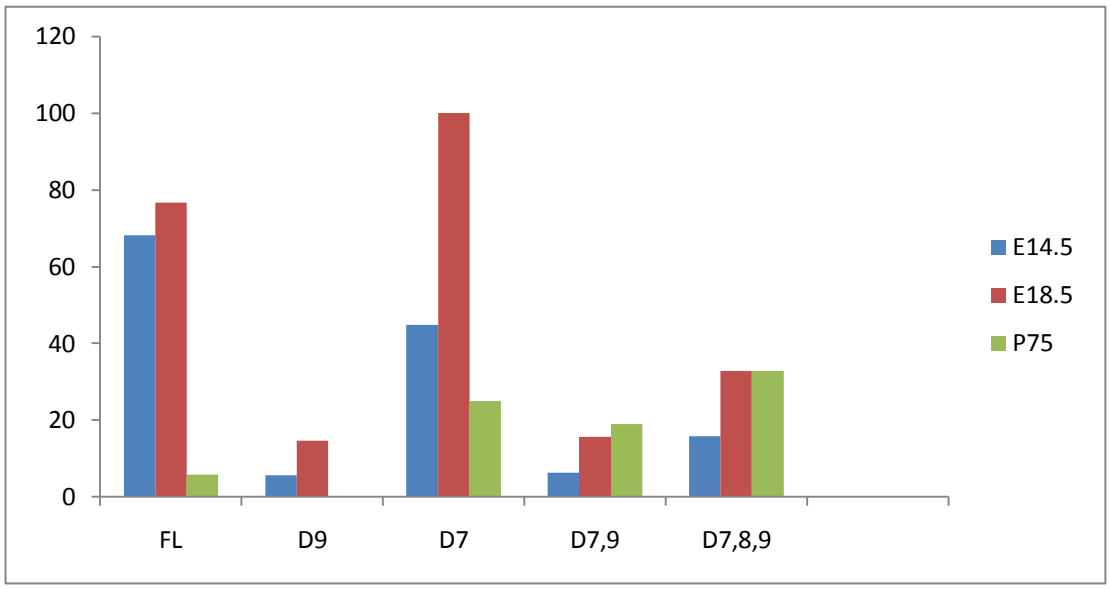


Figure 4. Identification of developmentally-regulated SUN1 Spliced Variants.

Two micrograms of total RNA extracted from E14.5, E18.5 and P75 mouse retinas were reverse transcribed and PCR-amplified. PCR was carried out for 35 cycles with 1 minute elongation. SUN1, primers were designed to surround the alternatively spliced region (between exon 3 and 10). PCR signals were quantified in non-saturating conditions. The relative abundance of each isoform was quantified (arbitrary units) for each time point.

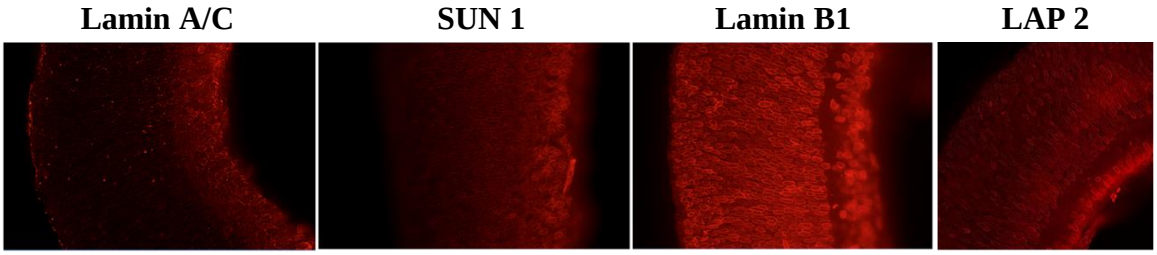


Figure 5. Early detection of LINC Complex components in P0 mouse retina.

Immunofluorescence staining of P0 retinas revealed a weak expression of both Lamin A/C and SUN1 in retinal precursor cells in comparison to Lamin B1 and LAP2.

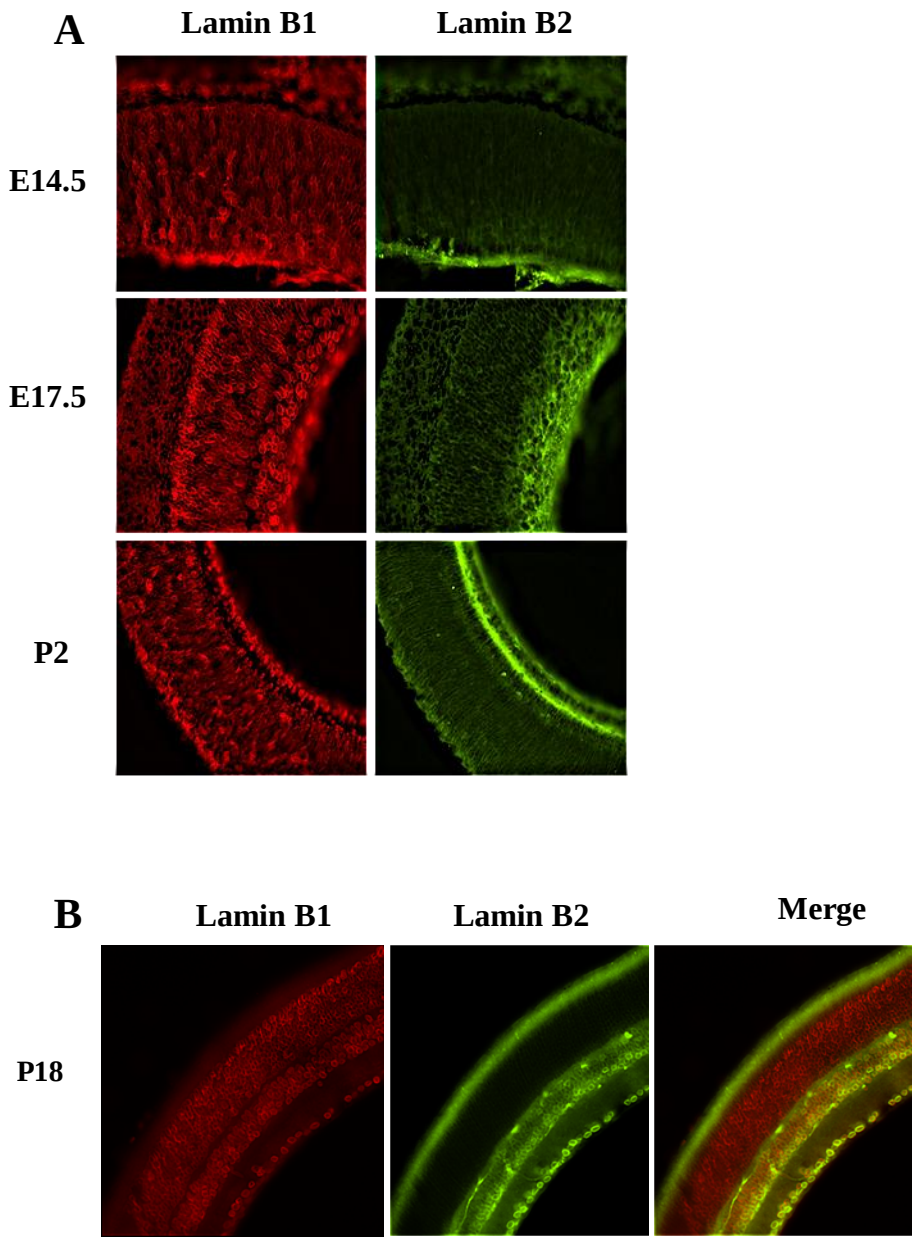


Figure 6. Lamin B1 and Lamin B2.

(A) Immunofluorescence staining of embryonic and postnatal retinas revealed that Lamin B1 is present in early embryonic stages and remains prevalent during postnatal development while Lamin B2 expression levels remain dormant. **(B)** Lamin B1 expression is evenly distributed throughout the three cell layers. By P12, Lamin B2 is expressed in the INL and RGC layers and is quite prominent by P18.

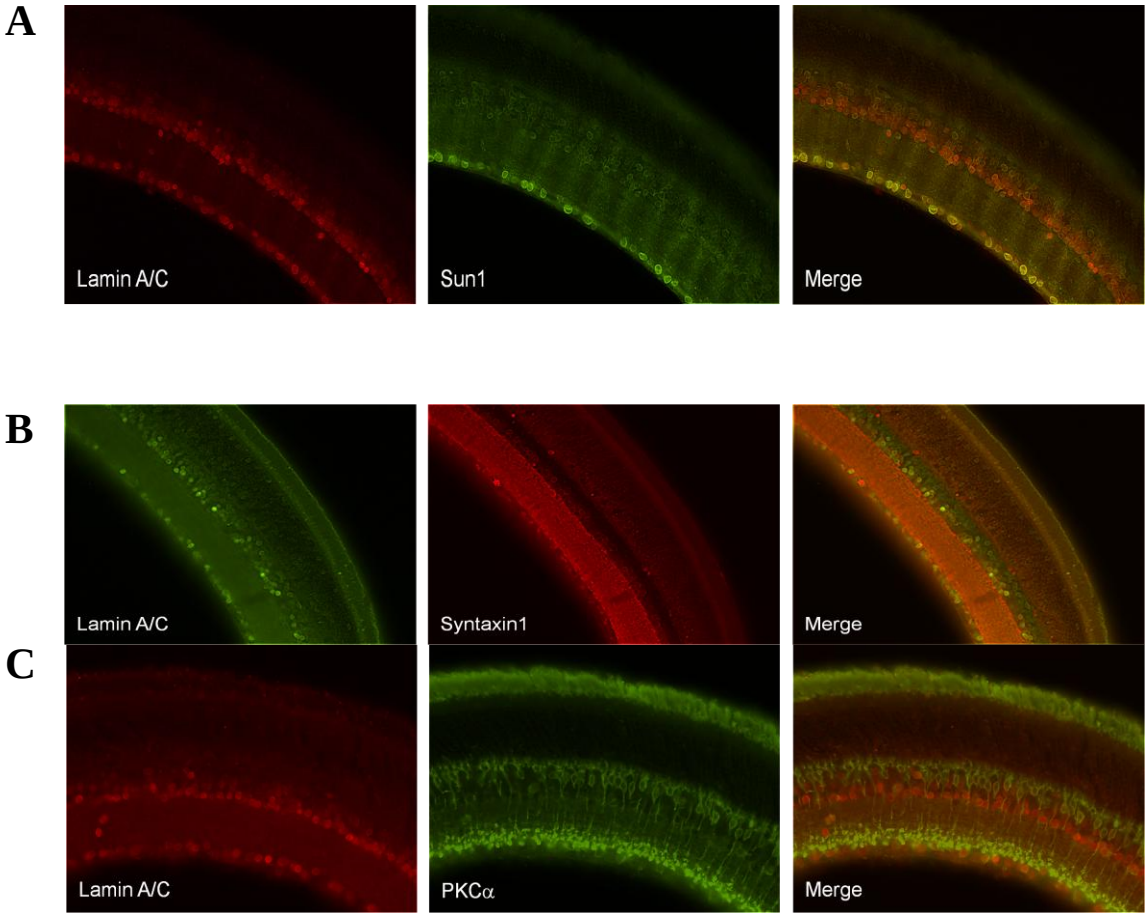


Figure 7. Immunolocalization of LINC Complex Components in mouse retina

(A). Immunolocalization of LINC Complex Components revealed that both Lamin A/C and SUN1colocolized in the INL and RGC layers. SUN1 shows a greater abundance in the RGC but is uniformally distributed over the entire INL. Weak expression of Lamin A/C and SUN1 were detected in rods but a more prominent signal was detected in cones. (**B**). Colocolization of Lamin A/C and amacrine cell marker Syntaxin1 revealed a preferential expression in amacrine cells within the INL. (**C**). Colocolization of Lamin A/C and rod bipolar cell marker PKCa revealed a paucity expression in rod bipolar cells within the INL.

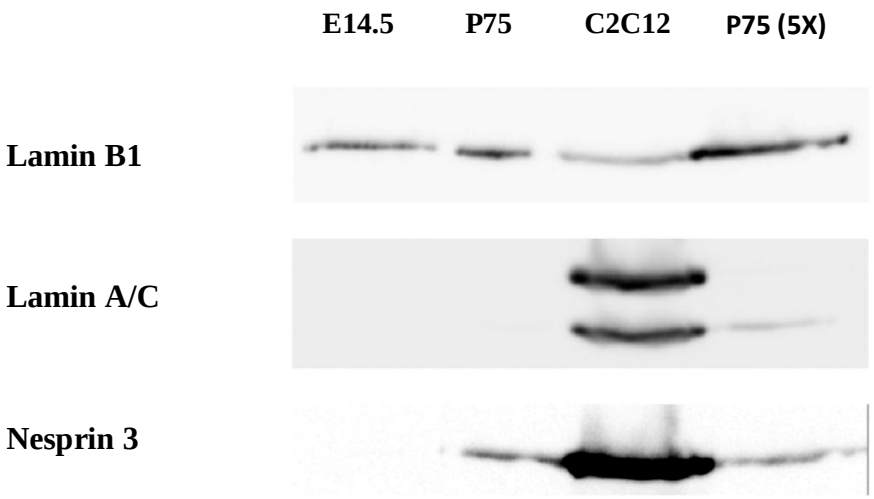


Figure 8. Western blot detection of LINC Complex components in mouse retina.

E14.5, P75 mouse retinas and C2C12 mouse myoblast cells were homogenized in laemli buffer. Equivalent amounts of E14.5 and P75 retina lysates were analyzed in Western blots with indicated antibodies. C2C12 lysates were used to confirm the identity of bands detected in retina lysates. Western blot analysis revealed constitutive expression of Lamin B1 and a weak expression of Lamin A/C and the absence of Nesprin 3 in embryonic retina as suggested by RT-PCR analysis.

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