

Nesprin-2 coordinates dynein and kinesin-1 for neuronal nuclear migration

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Table of Contents

Abstract.....	4
List of Abbreviation.....	5
1 Chapter 1 - Neuronal nuclear movements are driven by microtubule motors.....	6
1.1 Introduction	6
1.1.1 Bidirectional cargo transport driven by dynein and kinesin motors	6
1.1.2 Nucleus is transported by microtubule motors during neural development	7
1.2 Results	11
1.2.1 Both dynein and kinesin-1 are required for nuclear translocation during neuronal migration	11
1.2.2 Nuclear shape is not changed upon dynein or kinesin-1 inhibition	14
1.3 Discussion.....	15
2 Chapter 2 – Nesprin-2 functions as an adaptor for microtubule-motor-driven nuclear transport	18
2.1 Introduction	18
2.1.1 Nesprins mediate nucleo-cyskeletal interactions	18
2.1.2 Nesprin-2 is required for neuronal nuclear migration.....	19
2.2 Results	22
2.2.1 Nesprin-2 recruits dynein-dynactin-BicD2 complex and kinesin-1 through distinct but physically close motifs.....	22
2.2.2 Nesprin-2-deficient CGCs showed delayed nuclear migration during cerebellum development..	28
2.2.3 Nesprin-2 SR48-KASH rescues impaired nuclear migration by binding with both dynein and kinesin-1	31
2.2.4 Nesprin-2 generates persistent cargo movements bidirectionally along microtubules	33
2.2.5 Nesprin-2 mediates active cargo transport when bound with opposing motors	36
2.2.6 Nesprin-2-mediated cargo transport is not subject to size effects.....	42
2.2.7 Nesprin-2 generates persistent cargo transport bidirectionally in migrating CGCs	45
2.3 Discussion.....	47
2.3.1 Nesprin-2 is required for nuclear translocation in migrating neurons	47
2.3.2 Nesprin-2 interacts with dynein-dynactin-BicD2 complex and kinesin-1	48
2.3.3 Nesprin-2 supports sustained bidirectional cargo dynamics.....	49
2.3.4 Limitations and future directions	50
3 Chapter 3 – Nucleus is linked to forward-moving microtubules via Nesprin-2	51
3.1 Introduction	51
3.2 Results	53
3.2.1 Nuclear migration is correlated with microtubule forward movements during neuronal migration	53
3.2.2 Inhibition of Nesprin-2 disconnects nucleus and forward-moving microtubules.....	53
3.3 Discussion.....	56
4 Chapter 4 – Concluding thoughts and future outlook	57
5 Materials and Methods	59

5.1	Animals.....	59
5.2	Plasmids and reagents.....	59
5.3	Organotypic and primary culture of CGCs.....	61
5.4	Time-lapse imaging of CGCs	62
5.5	Isoform analysis and protein structure prediction	62
5.6	Cell line culture.....	63
5.7	Co-immunoprecipitation and Western Blot.....	63
5.8	Establishment of BicD2 knockout cell lines	65
5.9	Establishment of Nesprin-2 mutant mouse line.....	65
5.10	Immunohistochemistry and analysis of cerebellum morphology	66
5.11	Immunocytochemistry of CGC primary cultures	67
5.12	Drug-inducible peroxisome trafficking assay	67
5.13	Photoconversion experiments in CGCs.....	68
5.14	Image analysis and data presentation	68
5.15	Statistical tests	70
6	<i>Bibliography</i>	71
7	<i>Acknowledgements</i>	85

Abstract

Nuclear migration is critical for the proper positioning of neurons in the developing brain. It is known that the nucleus is transported by the minus- and plus-end-directed microtubule motors, dynein and kinesin-1, yet how bidirectional motors are coordinated for efficient transport is still under debate. Nesprin-2 is a component of the LINC (Linkage between Nucleoskeleton and Cytoskeleton) complex, spanning the outer nuclear membrane and interacting with microtubule motors to support microtubule-dependent movements of the nucleus. Using mouse cerebellar granule cells, I investigated the role of Nesprin-2 in mediating the interplay between dynein and kinesin-1 during neuronal nuclear migration. Unlike the conventional view that dynein is the major driving force for forward nuclear migration, I showed by live-cell imaging that nuclear movements require both dynein and kinesin-1 in a cooperative manner. Biochemical assays revealed the putative Spindly and CC1-box motifs as the binding sites for dynein-dynactin-BicD2 complex and the nearby LEWD motif for kinesin-1/Kif5 motors, which recruit the opposing motors non-competitively and mostly independently. Genetic ablation of Nesprin-2 caused significant delay in nuclear migration both *in vivo* and *in vitro*, which could be rescued by overexpression of a Nesprin-2 fragment bound to both motors, but not by its mutant forms bound to either dynein or kinesin-1 alone. These findings indicate that Nesprin-2 enables functional cooperation between opposing motors, which is essential for nuclear movement. Through an intracellular cargo trafficking assay, I further revealed that Nesprin-2-motor complex generates active bidirectional transport along microtubules over an extended duration of time. In contrast, the single-motor-bound Nesprin-2 mutants showed reduced transport toward both microtubule ends, providing further evidence for motor co-dependence. The bidirectional motilities of Nesprin-2 raises another question on how the forward biased transport of the nucleus is achieved. By tracking microtubule movements in migrating cerebellar granule cells, I showed that, instead of being transported on stationary microtubules, the nucleus is pulled toward migration direction by microtubules that are advancing forward. Inhibition of Nesprin-2 disconnects the nucleus from the forward-moving microtubule rails. Taken together, I discovered that Nesprin-2 serves as an important adaptor for nuclear translocation in migrating neurons by coordinating the activities of opposing microtubule motors and anchoring the nucleus onto forward-moving microtubules.

List of Abbreviation

BicD2	Bicaudal D cargo adaptor 2
CC1	Coiled coil 1
CGC	Cerebellar granule cell
CH	Calponin homolog
DCX	Doublecortin
DDB	Dynein-dynactin-BicD2
DHC	Dynein heavy chain
FKBP	FK506 binding protein
FRB	FKBP-rapamycin binding protein
GM130	Golgi matrix protein 130
HTT	Huntingtin
IB	Immunoblotting
i-GONAD	Improved genome-editing via oviductal nucleic acids delivery
INM	Interkinetic nuclear migration
INM	Inner nuclear membrane (full spelling is always included in the text to distinguish from interkinetic nuclear migration)
IP	Immunoprecipitation
JIP	c-Jun N-terminal kinase (JNK) interacting protein
KASH	Klarsicht/ANC-1/Syne-1 homology
KHC	Kinesin heavy chain
KLC	Kinesin light chain
LINC	Linker of nucleoskeleton and cytoskeleton
LIS1	Lissencephaly-1
LSD	Lipid droplet protein
MISO	Mixture of isoforms
mNG	mNeonGreen
MTOC	Microtubule organizing center
NM	Nucleo-microtubule
PEX	Peroxisomal biogenesis factor/ Peroxisomal assembly protein
RanBP	Ran binding protein
SR	Spectrin repeat
TPR	Tetratricopeptide repeat
TRAK	Trafficking kinesin protein

1 Chapter 1 - Neuronal nuclear movements are driven by microtubule motors

1.1 Introduction

1.1.1 Bidirectional cargo transport driven by dynein and kinesin motors

The positioning of intracellular vesicles and organelles relies on cytoskeleton-based transport, which lays the foundation for cell morphology, polarity, function and motility (Vale, 2003). Microtubules serve as highways for long-range directional delivery of intracellular cargoes. Microtubule-based transport is carried out by dynein and kinesin motors. Dynein family includes cytoplasmic dynein-1 and ciliary dynein-2, both of which move cargoes to the microtubule minus ends (Roberts et al., 2013). Kinesin superfamily includes 14 kinesin families, among which kinesin-1 is the conventional kinesin, and kinesin-1 moves cargoes to the microtubule plus ends (Hirokawa et al., 2009). Mounting evidence has shown that vesicles, organelles and other intracellular cargoes are simultaneously bound to dynein and kinesin motors. Due to their opposite walking directions, dynein and kinesin are thought to generate bidirectional movements of the transported cargo, which needs to be regulated precisely to achieve targeted delivery in the cell (**Figure 1-1. A**).

There are three classic scenarios for bidirectional cargo transport driven by dynein and kinesin: (1) selective recruitment or activation, (2) tug-of-war and (3) co-dependence. Selective recruitment or activation model assumes that either one kind of the motors are recruited to cargo or activated under certain temporal or spacial conditions in cells. In this circumstance, dynein and kinesin motors function independently, and inhibiting one motor does not affect cargo transport by the other motor, which was observed in phagosome transport (Sanghavi et al., 2018). Such clear division of labor sounds efficient and appealing, however, most native organelles purified from animal tissue are bound with both opposing motors, and motor interplay has been implied by functional perturbation experiments (Maday et al., 2012; Hancock, 2014; Cason and Holzbaur, 2022). Dynein and kinesin may simply hinder each other in a tug-of-war manner or coordinate through more sophisticated mechanisms. The tug-of-war model was first described in lipid droplet transport (Welte et al., 1998; Müller et al., 2008), and later in many other organelles as well including neuronal vesicles, endosome and melanosome (Hendricks et al., 2010; Soppina et al., 2009; Gross et

al., 2002). In this model, dynein and kinesin motors compete against each other and the “winning” motor dictates cargo transport direction, which might be energy-inefficient but provides flexibility for rapid direction reversal. One prediction from the tug-of-war model is that inhibition of one type of motor should enhance the transport driven by the opposing motor due to lessened resistance. However, contradictory results have been reported in the transport of peroxisome, mitochondria and many other intracellular cargoes, demonstrating that inhibiting one motor diminishes transport by the opposing motor (Ally et al., 2009; Fu and Holzbaur, 2013; Fenton et al., 2021). These results suggest a co-dependent interplay between dynein and kinesin. The mechanisms for regulating the selective activation, competition or cooperation between dynein and kinesin on different intracellular cargoes are still largely unknown.

1.1.2 Nucleus is transported by microtubule motors during neural development

The nucleus, the largest organelle in the cell, is also subject to microtubule-based transport and the positioning and movements of the nucleus are regulated by molecular motors. Nuclear movements in neurons have been extensively studied when nuclei of radial progenitor cells undergo interkinetic nuclear movements (INM) to produce neural progenitors or neurons in cerebral cortex. The exact purpose of such nuclear movements is still elusive, but it is known to play an important role in proper neurogenesis during development. In radial progenitor cells, the centrosome is located at the apical endfoot and emanates microtubules to form a cage-like structure around the nucleus with plus ends pointing toward the basal direction. During INM, kinesin-3 motor, Kif1A, is responsible for basal (away from centrosome) nuclear migration during G1 phase, and dynein-1 is responsible for apical (towards centrosome) nuclear migration during G2 phase (**Figure 1-1. B**; Tsai et al., 2010). It represents a scenario where motors are selectively activated at distinct cell cycle stage.

Directed nuclear movements also occur in neuronal migration. During vertebrate brain development, post-mitotic neurons undergo collective migration from their neurogenic sites to designated locations, which is essential for cortical layer formation and circuitry maturation. Migrating neurons are highly polarized, with cytoplasmic organelles such as the centrosome and the Golgi apparatus at their leading edge, and the nucleus in their middle or rear cell soma. The forward translocation of the nucleus is a critical step for neuronal migration, which was first described in detail by Rakic (1972) in the developing cerebral

cortex. Pioneering studies using the cerebellar granule cells (CGCs) identified perinuclear microtubule network which connects to microtubule bundles in the leading process, and proposed that the polarized microtubules may create forces for nuclear displacement (**Figure 1-1. C**; Rivas and Hatten, 1995; Rakic et al., 1996). Breakthrough was made with the discovery of the causal genes for type I lissencephaly, a heterogeneous group of disorders related to abnormal neuronal migration affecting cortical formation. Lissencephaly-1 (LIS1) was identified as the causal gene product, which binds to the motor domain of cytoplasmic dynein and regulates dynein-dependent transport of the nucleus along intracellular microtubule tracks (Hirotsume et al., 1998; Tanaka et al., 2004; Shu et al., 2004). Afterwards, more dynein-related mutations were linked to neuronal migration defects, contributing to the common view that the cytoplasmic dynein-1 complex (dynein hereafter) drives forward nuclear translocation in migrating neurons (Ayala et al., 2007; Tsai and Gleeson, 2005). Since dynein transports cargoes toward microtubule minus ends, it is in line with the observation that most peri-nuclear microtubules have their minus ends pointing forward (**Figure 1-1. C**; Tsai et al., 2007). However, like many other intracellular organelle transport and INM, the nucleus in migrating neurons was found to recruit the opposing motor, kinesin-1 as well, raising an intriguing question that how the activities of kinesin-1 and dynein are regulated for one-way nuclear translocation. On one hand, Gonçalves et al (2020) reported that, during radial migration of rat cortical neurons, kinesin-1 retards forward nuclear translocation by tug-of-war competition with dynein and inhibition of kinesin-1 accelerates nuclear displacement (**Figure 1-1. C**). On the other hand, a study by our group revealed that, while dynein functions as a dominant motor, kinesin-1 motor generates nuclear rotation, assisting forward translocation of the nucleus, and inhibition of kinesin-1 decreases forward nuclear displacement during cell migration in *in vitro* culture of mouse CGCs (**Figure 1-1. C**; Wu et al., 2018). Wu et al. (2018) also reported that peri-nuclear microtubules are of mixed polarity with 80% of the microtubules oriented with their minus ends forward (**Figure 1-1. D**), which further adds to the complexity of directional control for nuclear movements. A consensus has not been reached on whether kinesin-1 plays an inhibitory or assistive role in dynein-dominating nuclear transport.

In this chapter, I used both organotypic and *in vitro* culture of mouse CGCs to perform time-lapse observation of dynamic nuclear movements during cell migration. Through inhibition with dominant negative molecules, I found that directional nuclear migration requires both

dynein and kinesin-1, and novel mechanisms need to be proposed to explain how opposing motors work together to achieve forward nuclear translocation

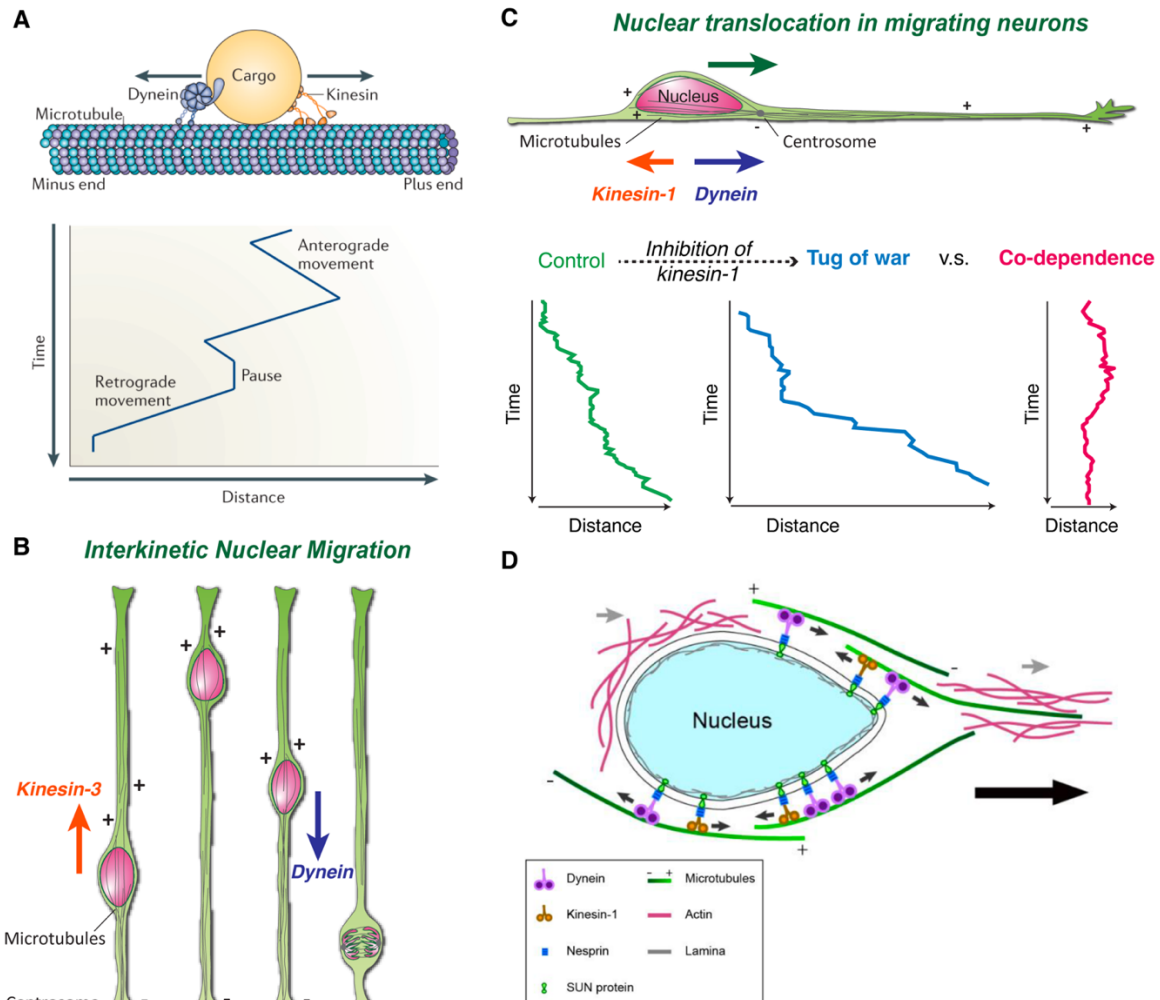


Figure 1-1. Bidirectional cargo transport is carried out by dynein and kinesin. (A) Schematic of bidirectional cargo transport and a representative trajectory of cargo movements. Adapted from Hancock (2014). **(B)** Schematic of interkinetic nuclear migration in radial progenitor cells. Plus (+) and minus (-) ends of microtubules are indicated and centrosome is shown. Kinesin-3 drives basal-directed nuclear movements toward microtubule plus ends while dynein drives apical-directed nuclear movements toward minus ends. Cell division takes place when the nucleus reaches the apical side. **(C)** *Upper*, schematic of nuclear translocation in a migrating neuron with microtubule ends and centrosome indicated. *Bottom*, trajectories of nuclear movements in control situation or upon kinesin-1 inhibition. When kinesin-1 is inhibited, tug-of-war model predicts accelerated dynein-driven transport (indicated in blue) and co-dependence model predicts reduced movements toward both directions (indicated in magenta). **(D)** An updated mechanistic view of nuclear movements in mouse CGCs. Microtubule motors facilitate directional nuclear migration via Nesprin-SUN

linker molecules. Actin provides extra pushing and pulling forces to further support nuclear movements. Adapted from Wu et al. (2018).

1.2 Results

1.2.1 Both dynein and kinesin-1 are required for nuclear translocation during neuronal migration

I utilized organotypic culture of mouse cerebellum lobes to examine the dynamics of nuclear translocation during neuronal migration. By *in vivo* electroporation on the cerebellum of postnatal mice, I transfected the CGCs with EGFP as a cell volume marker and DsRed-NLS as a nuclear marker. Organotypic culture of cerebellar lobes was prepared one day after electroporation and live-cell imaging of 30-sec intervals was performed to reveal nuclear movements at a high spatiotemporal resolution (**Figure 1-2. A**). I found that the nucleus advanced forward with occasional backward steps (**Figure 1-2. B, E**). When dynein function was inhibited by overexpression of the N-terminal truncate of a dynein complex accessory protein Lis1 (Lis1N), nuclear movements were greatly diminished (**Figure 1-2. C, E-H**), consistent with the conventional consensus that dynein drives forward nuclear translocation in migrating neurons. If dynein functions as a predominant motor and transports the nucleus in a tug-of-war competition with kinesin-1, kinesin-1 inhibition should accelerate the forward migration of the nucleus as previously observed in neocortical migrating neurons (Gonçalves et al., 2020). However, overexpression of the tail domain of kinesin-1 heavy chain (Kif5B-tail) as a dominant negative failed to accelerate nuclear movement, but rather strongly inhibited it in CGCs (**Figure 1-2. D-H**). I measured the nuclear displacement from the initial and final positions of the nucleus, as well as the absolute distance that the nucleus has traveled, including forward and backward distance. Both measurements suggest that kinesin-1 assists the forward nuclear transport instead of competing against dynein.

I further validated the functional involvement of dynein and kinesin-1 motors in primary culture of CGCs from postnatal mice. The migratory behaviors of CGCs can be reconstituted *in vitro* in a reaggregate culture (**Figure 1-3. A**; Umeshima et al., 2007). At 1~2 days in culture, each CGC extended a long leading process out of the cell aggregate and delivered the nucleus forward with frequent backward steps (**Figure 1-3. A-C**). Consistent with the previous report by Wu et al. (2018), I observed decreased nuclear migration upon the inhibition of dynein by overexpression of either Lis1N or the coiled-coil 1 of dynactin p150 (p150-CC1), or upon the inhibition of kinesin-1 by overexpression of Kif5B-tail or the tetratricopeptide repeat domain of kinesin light chain 1 (KLC1-TPR) (**Figure 1-3. B-H**).

These results further support that kinesin-1 synergistically works with dynein to generate nuclear movements, rather than engaging in a tug-of-war.

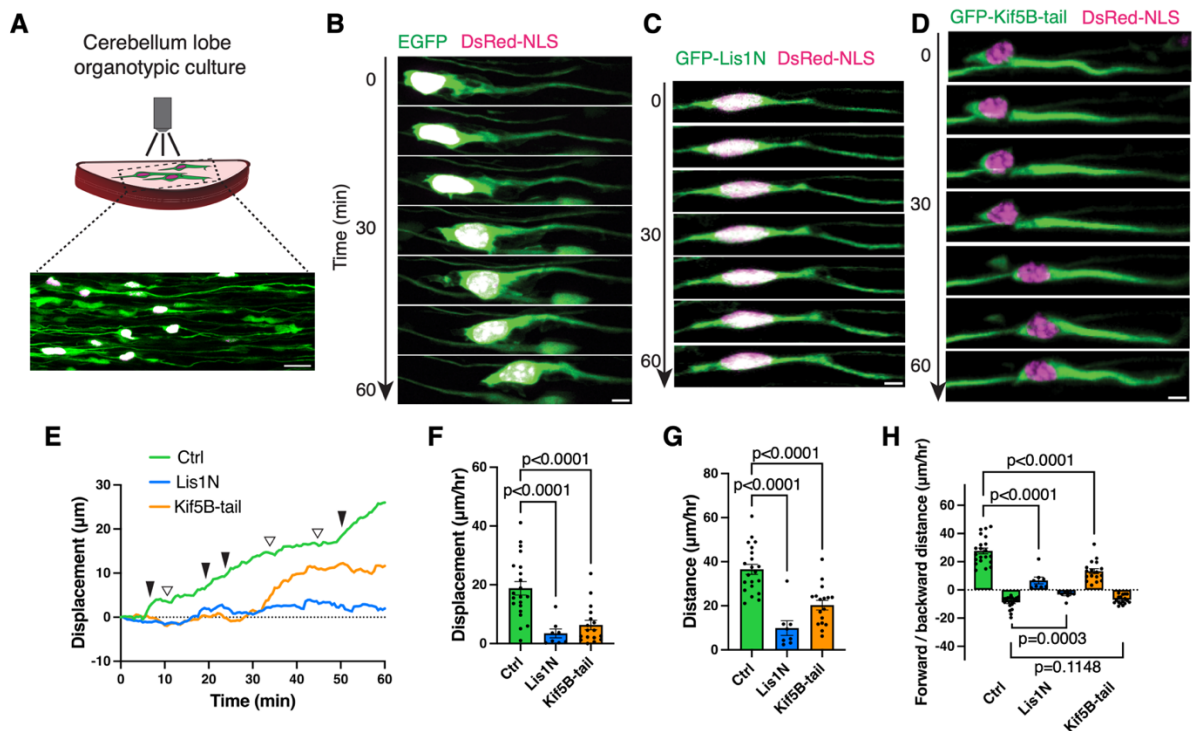


Figure 1-2. Nuclear migration in lobe culture was impeded upon dynein or kinesin-1 inhibition. (A) Schematic of cerebellar lobe culture. Cells were transfected by in vivo electroporation. A lobe was isolated from cerebellum and plated flat to observe tangential migration parallel to the pial surface. (B-D) Time series of representative CGCs transfected with EGFP (B), GFP-Lis1N (C) and GFP-Kif5B-tail (D) respectively in lobe culture. DsRed-NLS (*magenta*) was used to label the nuclei. (E) Trajectories of nuclear centroid movements of CGC transfected with GFP (Ctrl; *green*), GFP-Lis1N (*blue*) or GFP-Kif5B-tail (*orange*). Filled arrowheads indicate forward steps. Hollow arrowheads indicate backward steps. (F-H) Quantitative analysis of CGC nuclear net displacement (F), total travel distance (G) and total forward/backward travel distance (H). n = 21 (Ctrl), 8 (Lis1N) and 17 (Kif5B-tail) neurons from 3 independent experiments per group. Unpaired t test with Welch's correction. Bars show mean ± SEM. Scale bars, 20 μm in (A); 5 μm in (B-D).

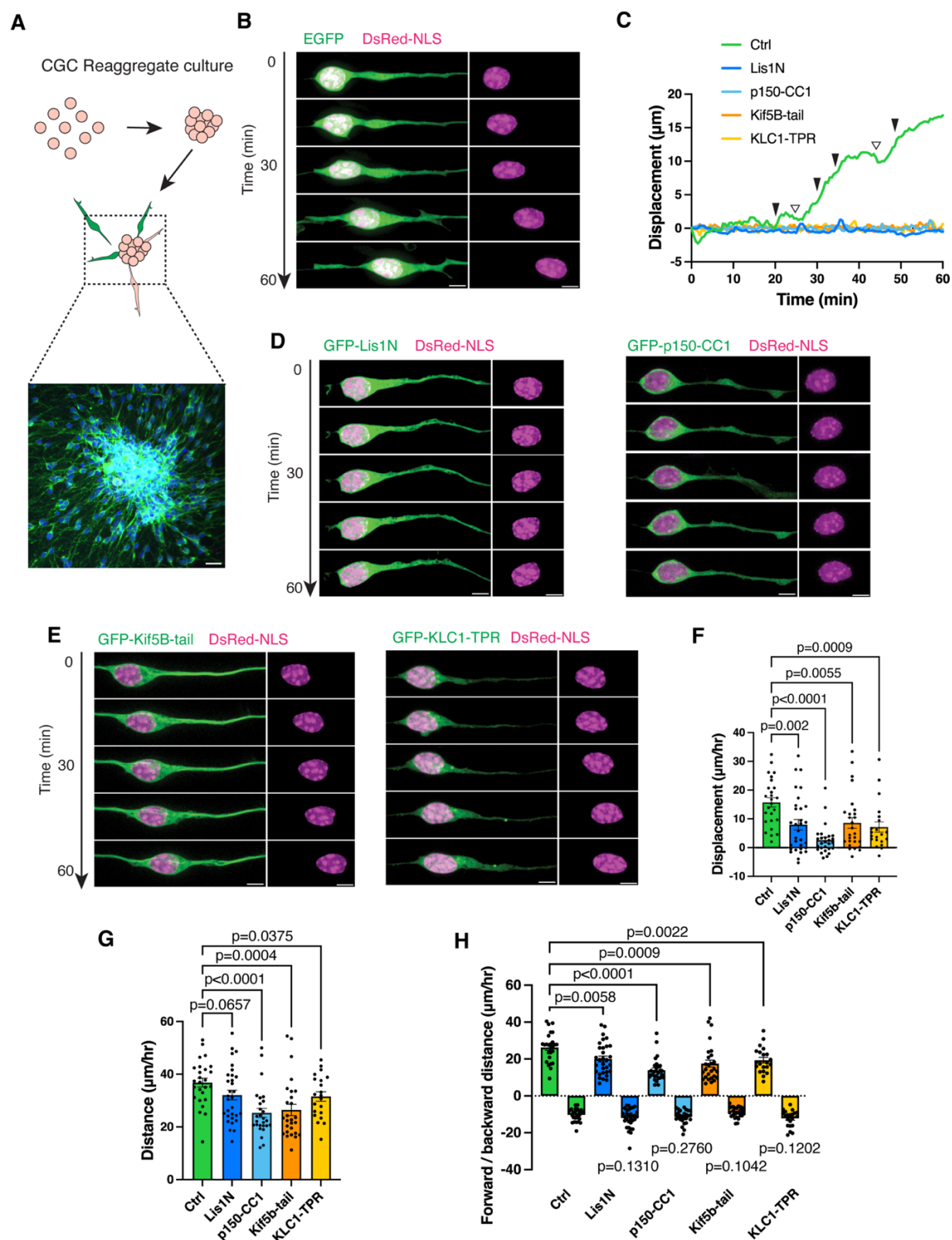


Figure 1-3. Nuclear migration in reaggregate culture was impeded upon dynein or kinesin-1 inhibition. (A) Schematic of CGC reaggregate culture. CGCs were isolated from cerebellum, digested into single cells and then incubated at a high density to form reaggregates. After plating the reaggregates on coated surface, CGCs migrated out from reaggregates. (B, D-E) Time series of representative CGCs transfected with EGFP (B), GFP-Lis1N (the left panel in D), GFP-p150-CC1 (the right panel in D), GFP-Kif5B-tail (the left

panel in E) and GFP-KLC1-TPR (the right panel in E) respectively in reaggregate culture. DsRed-NLS (*magenta*) was used to label the nuclei. **(C)** Trajectories of nuclear centroid movements of CGC transfected with EGFP (Ctrl; green), GFP-Lis1N (blue), GFP-p150-CC1 (cyan), GFP-Kif5B-tail (orange) or GFP-KLC1-TPR (yellow). Filled arrowheads indicate forward steps. Hollow arrowheads indicate backward steps. **(F-H)** Quantitative analysis of CGC nuclear net displacement (F), total travel distance (G) and total forward/backward travel distance (H). $n = 26$ (Ctrl), 31 (Lis1N), 27 (p150-CC1), 27 (Kif5B-tail) and 21 (KLC1-TPR) neurons from 3 ~ 4 independent experiments per group. Unpaired t test with Welch's correction was used to compare with Ctrl. Bars show mean $\pm$ SEM. Scale bars, 20 μ m in (A); 5 μ m in (B, D-E).

1.2.2 Nuclear shape is not changed upon dynein or kinesin-1 inhibition

Drastic shape changes can occur when the nucleus is moved by microtubule motors (Wu and Kengaku, 2018). It has been demonstrated that unbalanced force application by dynein and kinesin-1 could induced a sharp peak on the nucleus (Wu et al., 2018). Elongated shape of the nucleus is associated with active force application, while a rounder nucleus implies less force application. Therefore, I analyzed the aspect ratio as another indicator of the activities of molecular motors bound to the nuclear envelope (**Figure 1-4. A**). However, the results suggested no significant difference in nuclear shapes between control, Lis1N-, p150-CC1-, Kif5B-tail- or KLC1-TPR-overexpressing CGCs (**Figure 1-4. B**).

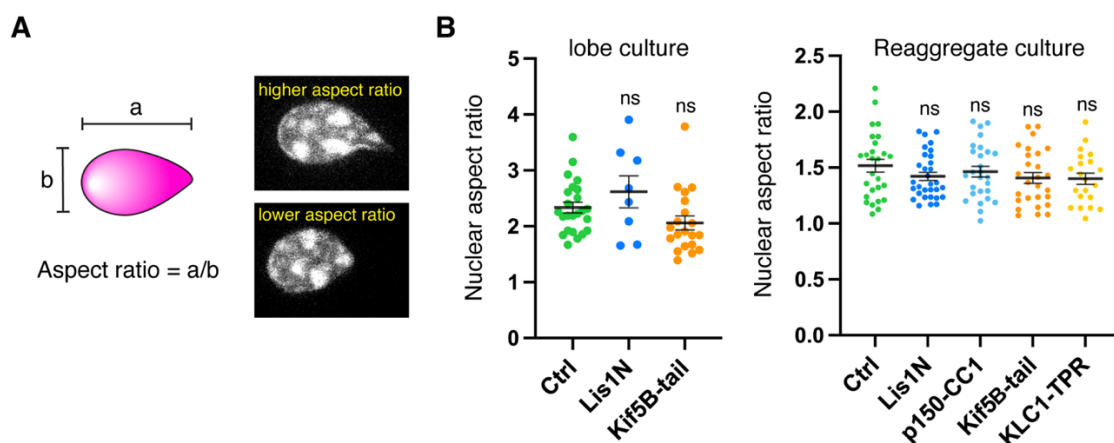


Figure 1-4. Nuclear shape did not show significant changes upon dynein or kinesin-1 inhibition. **(A)** Schematic of aspect ratio calculation. *Left*, nuclear aspect ratio is the value of the major axis (denoted as a) divided by the minor axis (denoted as b). *Right*, examples of CGC nuclei with a high or low aspect ratio. **(B)** Quantitative analysis of nuclear aspect ratio in lobe culture and reaggregate culture of CGCs. CGCs were transfected with EGFP (Ctrl; green), Lis1N (blue), p150-CC1 (cyan), Kif5B-tail (orange) or KLC1-TPR (yellow). The

aspect ratio is the average values from time-lapse imaging data for each cell. Unpaired t test with Welch's correction was used to compare with Ctrl. Bars show mean $\pm$ SEM.

1.3 Discussion

Together with the previous study by Wu et. al. (2018), my results showed that the movements of nucleus were greatly impeded upon inhibition by either dynein and kinesin-1 dominant negatives *in vivo* and *in vitro*. It suggests that opposing motor kinesin-1 is supporting dynein-dominant forward nuclear transport, which might be an underappreciated mechanism in neurodevelopmental diseases. Many developmental brain disorders resulting from defective neuronal migration have been linked to mutations in dynein or its accessory proteins like LIS1 (Feng and Walsh, 2001). Although there are studies showing that kinesin inhibition causes severe defects in neuronal cargo transport, the embryonic lethality of constitutive Kif5B knockout and functional redundancy of other Kif family members limits mechanistic study during neuronal migration (Tanaka et. al., 1998; Kanai et. al., 2000; Falzore et. al., 2009; Cromberg et. al., 2019). Mutations of Kif5 have also been associated with malformations of cortical development (MCD) and microcephaly in human patient (Poirier et al., 2013; Duquesne et al., 2020). Further research is needed to understand the role of kinesin in neuronal migration under physiological and pathological conditions.

In the scenario of bidirectional cargo transport, my results support the co-dependence mechanism between dynein and kinesin-1 in migrating CGCs. It is distinguished from the selective activation model of INM in radial progenitor cells, where dynein and kinesin-3 alternate to drive nuclear movements at opposite directions, or the tug-of-war model of nuclear migration in cortical neurons, where kinesin-1 slows down the dynein-driven forward transport. During INM, kinesin-3 inhibition only affects basal-directed migration (Tsai et al., 2010). During radial migration of rat cortical neurons, kinesin-1 inhibition accelerates forward nuclear translocation (Gonçalves et al., 2020). In the case of migrating mouse CGCs, I found that forward nuclear movements are inhibited by functional perturbation of either dynein or kinesin-1, although backward movements are largely unaffected. While my results suggest a different conclusion from previous studies of neuronal nuclear movements, differences in neuronal types and developmental stages might be part of the reasons for the discrepancy. The functional involvement of kinesin-1 in promoting dynein-dominating

transport raises an intriguing hypothesis that opposing microtubule motors work in a co-dependent manner for generating forward nuclear migration.

Hancock (2014) proposed three possible mechanisms for how opposing microtubule motors function co-dependently (**Figure 1-5**): (1) Microtubule tethering: opposing motor keeps the cargo near microtubules, enhancing the processivity of transport; (2) Mechanical activation: opposing mechanical force is required to activate transport; (3) Steric disinhibition: opposing motor releases steric auto-inhibition. Here, I also propose another mechanism which requires validation by future experiments: (4) Track searching: opposing motor generates small backward steps or rotation to untangle the cargo from roadblock and to enable smooth transport through crowded cytoplasm, which might be important for cargo with a big size like the nucleus. Although the overall backward movements and nuclear shape do not change significantly upon motor inhibition, there maybe local curvature changes on the surface of nuclear envelope where motor-induced point forces are applied. Due to the complexity of microtubule network around the nucleus, multiple motors acting simultaneously and complicated nuclear dynamics like rotation and shape chaging, it is challenging to determine which mechanism is in play by microscopic inspection alone. Further computational analysis or simulation with biophysical modelling will be needed for tackling the co-dependence paradox.

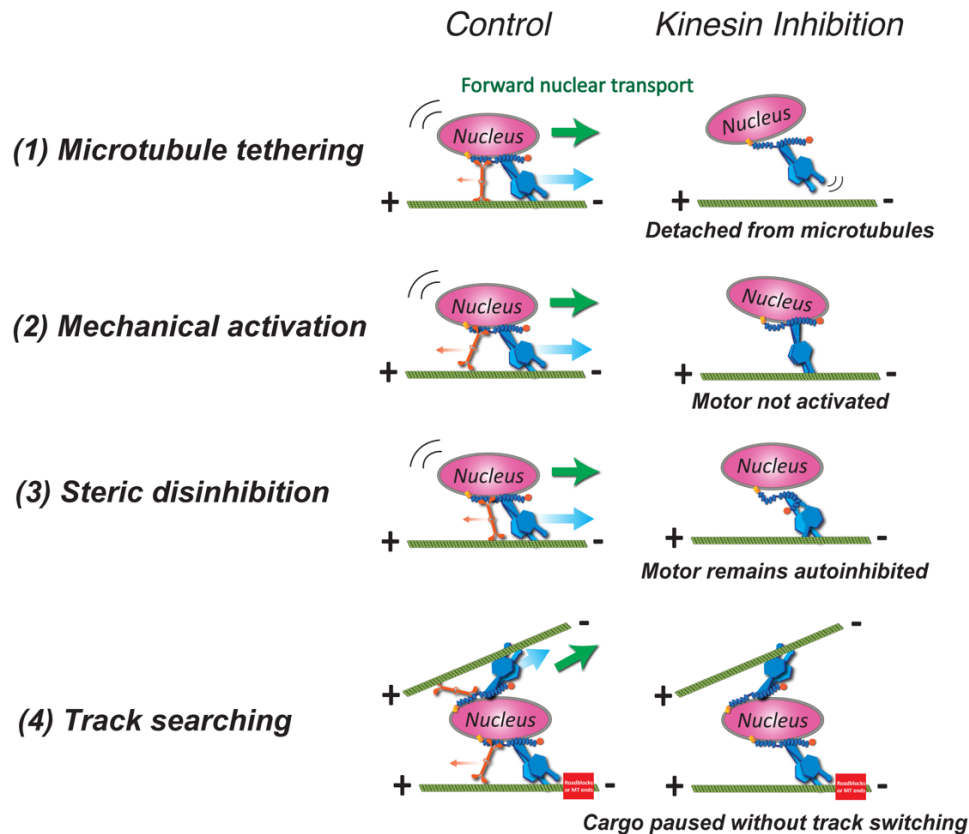


Figure 1-5. Mechanistic models for co-dependence of opposing microtubule motors.

From top to bottom, schematic mechanisms on how dynein and kinesin-1 transport the nucleus cooperatively are shown. (1) Dynein (blue and cyan) and kinesin-1 (orange) anchor the nucleus onto microtubules to generate dynein-dominant transport. When kinesin-1 is inhibited, the nucleus can not attach on microtubules efficiently. (2) The plus-end-directed opposing force induced by kinesin-1 mechanically activate minus-end-directed transport by dynein. When kinesin-1 is inhibited, dynein motor activity can not be initiated. (3) Dynein, kinesin-1 and potential adaptor proteins (shown as an extended linker in blue) between nucleus and motors form a complex that is optimal for dynein-dominant transport. When kinesin-1 is inhibited, dynein motor and adaptors remains in a auto-inhibitory conformation. (4) Kinesin-1-driven backward stepping helps dynein to avoid microtubule tracks with obstacles and to explore other available tracks for switching routes. When kinesin-1 is inhibited, dynein-driven transport pauses more frequently. Adaptor molecules mediating anchorage of motors onto the nucleus are also shown and may also contribute to the force transmission, conformation arrangement or coordinated activities of opposing motors.

2 Chapter 2 – Nesprin-2 functions as an adaptor for microtubule-motor-driven nuclear transport

2.1 Introduction

2.1.1 Nesprins mediate nucleo-cyskeletal interactions

Coordinated bidirectional transport of various cargos is believed to be facilitated by a diverse array of cargo-specific adaptors. These adaptors not only establish physical connections between cargos and motors but also regulate motor activities (Maday et al., 2014; Cason and Holzbaur, 2022). There are some well-characterized adaptors, including TRAK1/2 (trafficking kinase prtein 1/2) for mitochondria transport, Hook1-3 for endosome and Golgi transport, JIP1-4 (c-jun N-terminal kinase interacting protein family) for axonal vesicle transport, and HTT (Huntingtin) for synaptic vesicle precursor transport (Van Spronsen et al., 2013; Fenton et al., 2021; Canty et al., 2023; Bielska et al., 2014; Walenta et al., 2001; Olenick et al., 2016; Fu and Holzbaur, 2013; Fu et al., 2014; Kelkar et al., 2005; Colin et al., 2008). Many studies have elucidated the multifaceted roles of various adaptor proteins. However, a comprehensive understanding of the mechanisms underlying bidirectional organelle transport remains elusive.

It is known that the LINC (linker of nucleoskeleton and cytoskeleton) complex, which are embedded in the nuclear membrane, serve as cargo adaptors linking microtubule motors to the nucleus. Klarsicht/ANC-1/Syne homology (KASH) domain proteins are the components of the LINC complex which span the outer nuclear membrane and interact with actin and microtubule motors (**Figure 2-1**). The Drosophila KASH protein, Klarsicht (also known as *marbles*), is among the first KASH proteins to be characterized and it plays an important role in eye development by regulating apical nuclear migration (Mosley-Bishop et al., 1999). The vertebrate KASH proteins, Nesprins, are known to control nuclear positioning and shape, nuclear migration, chromatin organization, and mechanotransduction under physiological and pathological conditions (Razafsky and Hodzic, 2009; Mellad et al., 2011; Gundersen and Worman, 2013). There are five members in Nesprin familiy (**Figure 2-1**): (1) Nesprin-1, -2 and -3 are ubiquitously expressed in various cell types. Both Nesprin-1 and -2 bind to kinesin and dynein motors. Nesprin-1 is involved in cytokinesis by recruiting kinesin-2/Kif3B (Fan and Beck, 2004). Knockout of Nesprin-2 leads to neuronal migration defects in mouse

cerebral cortex while knockout of Nesprin-1 does not show obvious brain defects (Zhang et al., 2009); (2) Nesprin-3 interacts with intermediate-filament-associated proteins, plectin and vimentin, for structural support (Wilhelmsen et al., 2005; Ketema et al., 2013); (3) Nesprin-4 is expressed in sensory hair cells and secretory epithelial cells, and interacts with kinesin-1 for nuclear positioning control (Taiber et al., 2022); (4) KASH5 interacts with dynein and regulates meiosis in reproductive organ (Agrawal et al., 2022). Based on the involvement of dynein and kinesin-1 in coordinated nuclear transport, Nesprin-2 is the most likely candidate for mediating such function in migrating CGCs.

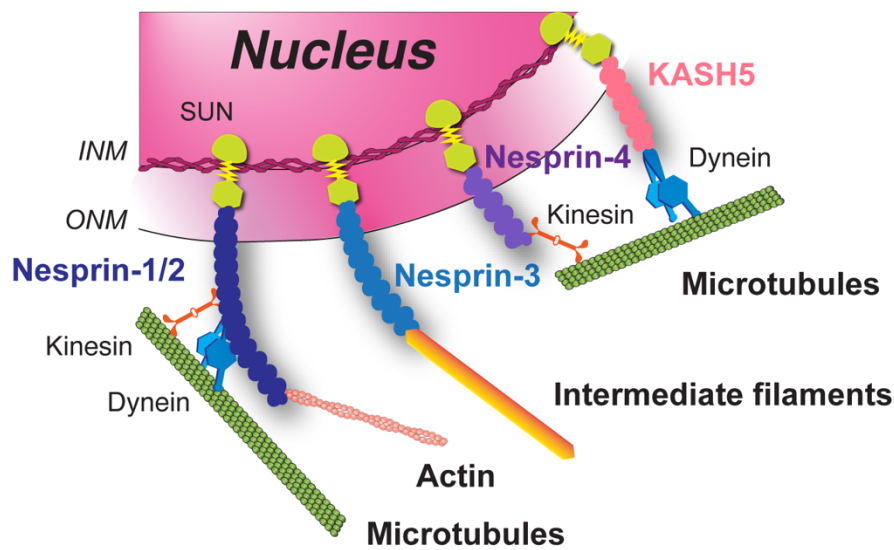


Figure 2-1. LINC complexes connect nucleoskeleton and cytoskeleton. The nucleus is encapsulated by the nuclear envelope, which is composed of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM). INM-localizing SUN proteins and ONM-localizing KASH proteins form LINC complexes. Nesprin-1/2 (dark blue) binds to microtubules via kinesin and dynein motors, and interacts with actin as well. Nesprin-3 (light blue) binds to intermediate filaments. Nesprin-4 (purple) binds to kinesin. KASH5 (pink) binds to dynein.

2.1.2 Nesprin-2 is required for neuronal nuclear migration

Accumulating evidence supports that Nesprin-2 serve as a cargo adaptor anchoring dynein and kinesin-1 to the nucleus in migrating neurons (**Figure 2-2**). Nesprin-2 (also known as Nesprin-2 Giant or Nesprin-2G) is a huge protein composed of 56 spectrin repeats (SRs) flanked by N-terminal actin-binding calponin homology (CH) domains and a C-terminal

KASH domain (**Figure 2-3. A**). Nesprin-2 directly interacts with kinesin-1 via the LEWD (Leu-Glu-Trp-Asp) motif in the nuclear membrane proximal region of their long cytoplasmic stretches (Wilson and Holzbaur, 2015). Nesprin-2 interacts with dynein-dynactin complex by unknown mechanisms possibly involving dynein associated protein, BicD2 (Bicaudal D2) (Zhang et al., 2009; Gonçalves et al., 2020; Tsai et al., 2020). It is still unclear how Nesprin-2 coordinates the binding and/or activity of the opposing motors.

In this chapter, I showed that dynein binding to Nesprin-2 requires the dynein-dynactin interacting CC1-box and Spindly motifs in the membrane proximal region, independently of kinesin-1 binding to the nearby LEWD motif. By using an intracellular cargo trafficking assay, I demonstrated that Nesprin-2 drives active bidirectional transport along microtubule tracks, which persists for a longer period than dynein- or kinesin-1-driven transport. Moreover, the mutant Nesprin-2 molecules that are defective of binding to either motor not only attenuate the motor-dependent transport, but also impair the activity of its opposing motor, suggesting cross-functional activation by opposing motors.

Several studies have reported mouse models with Nesprin-2 knockout, but the observed phenotypes varied, including defects in brain development, retinal layer formation, neuromuscular junctions, and slower wound healing, depending on the knockout methods used (Zhou et al., 2018). Although Nesprin-2 knockout mice showed impaired cerebral cortex formation linked to defected neuronal migration, it was reported that cerebellum development remained unaffected, except for a slightly reduced size (Zhang et al., 2009). Here, I established a new mouse model with Nesprin-2 deficiency and re-examined the impaired CGC migration during cerebellum development.

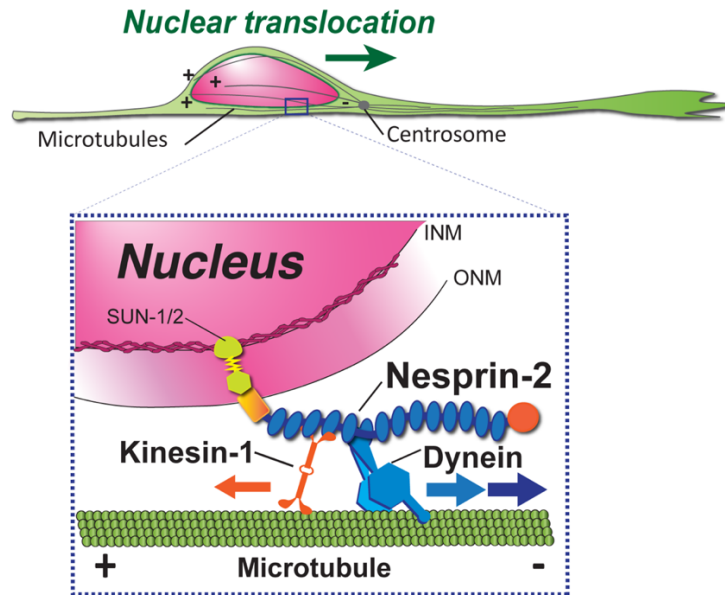


Figure 2-2. Nesprin-2 functions as an adaptor for microtubule-motor-driven nuclear movements in migrating CGCs. *Top*, a migrating CGC with perinuclear microtubules. *Bottom*, an enlarged view showing that Nesprin-2 is anchored in the outer nuclear membrane (ONM) through association with SUN-1/2 in the inner nuclear membrane (INM). Nesprin-2 mediates microtubule-based nuclear transport by recruiting dynein and kinesin-1.

2.2 Results

2.2.1 Nesprin-2 recruits dynein-dynactin-BicD2 complex and kinesin-1 through distinct but physically close motifs

Since Nesprin-2 has a repertoire of isoforms transcribed from the same gene *Syne2*, I first searched for the dominant form expressed in migrating CGCs. Using bulk RNA sequencing of postnatal cerebellar granule cells, I performed the mixture-of-isoform analysis (MISO) based on Katz et al. (2010). The full-length transcripts of Nesprin-2 occupied around half of the total *Syne2* transcripts, while several C-terminal isoforms of Nesprin-2 are also present at relatively high levels (**Figure 2-3. B**). I suspected that the potential dynein-interacting regions might be near the C-terminus. Recent studies characterized two protein sequence motifs that recruits dynein and dynactin complexes, which are Spindly motifs (xLF/AxE where x denotes any amino acid) and CC1-box motifs (AAxxG). Throughout the full-length Nesprin-2, several candidate dynein-interaction sites were identified by sequence alignment with Spindly and CC1 box motifs (**Figure 2-3. A, C**). I then designed a series of Nesprin-2 truncates spanning the full length of Nesprin-2 SR1-56 for co-immunoprecipitation (co-IP) in HEK293T cell lysate to examine possible interactions with dynein and kinesin-1 motors (**Figure 2-4. A**). Among the six truncates that was cloned, the most membrane-proximal fragment (SR48-56), which contains three potential Spindly motifs, one potential CC1-box motif, and the reported LEWD motif, was sufficient for binding to dynein heavy chain (DHC), the dynactin subunit p150^{Glued}, and kinesin heavy chain (KHC) (**Figure 2-4. B, C**). SR48-56 also recruited the dynein activator BicD2, which has been reported to mediate Nesprin-2 and dynein-dynactin interactions (Gonçalves et al., 2020). Other fragments showed only weak interactions to both motors and motor-associated proteins.

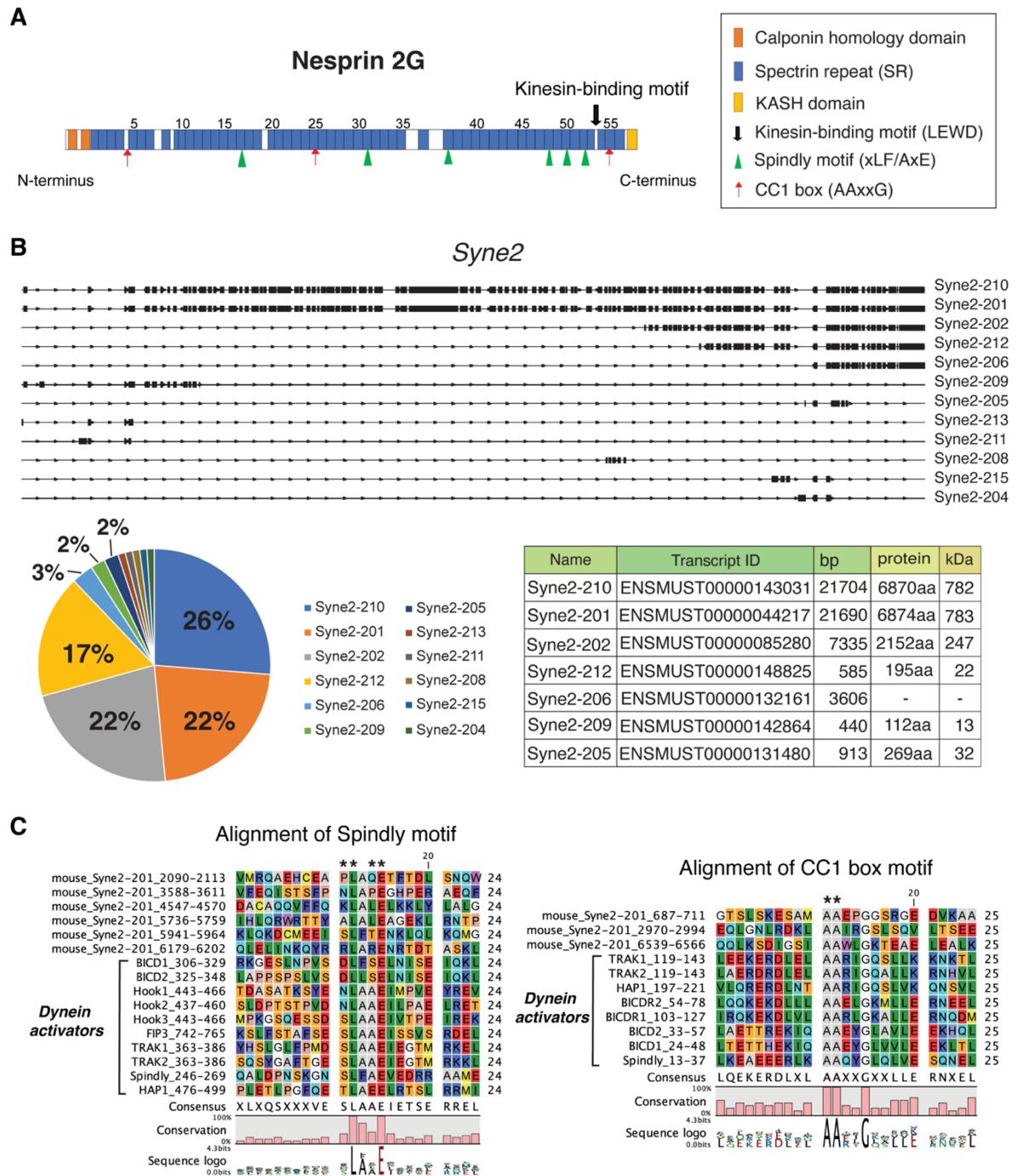


Figure 2-3. Isoform analysis and sequence search for dynein binding sites on Nesprin-2. (A) Schematic of Nesprin-2G with the kinesin-1-binding motif and the potential dynein-binding motifs highlighted. (B) The relative abundance of Nesprin-2 transcripts analyzed by MISO. Both Syne2-210 and Syne2-201 transcripts are translated as the full-length Nesprin-2G protein. Syne2-206 undergoes nonsense mediated decay. (C) Sequence alignment of the putative Spindly motifs (left) and CC1 box motifs (right) of Nesprin-2, together with other known dynein adaptors. Asterisks indicate the conserved amino acids within the motifs. (C) is adapted from Olenick and Holzbaur (2019).

I next prepared the construct containing the SR48-56 and the nuclear-localizing KASH domain (SR48-KASH), together with a series of its truncates to compare their binding affinities to the dynein-dynactin-BicD2 (DDB) complex and kinesin-1 by co-IP. SR49-KASH, lacking the SR48 domain compared to SR48-KASH, and SR52-KASH, lacking another three SRs, contained two and one putative Spindly motifs/motif respectively. Remarkably, SR48-KASH, SR49-KASH and SR52-KASH exhibited progressively reduced binding to both the DDB complex and kinesin-1 (**Figure 2-4. D, E**). Previous studies showed that SR52-KASH is capable of binding to both the dynein-dynactin complex and kinesin-1 to generate microtubule-based nuclear movements (Zhu et al., 2017; Gonçalves et al., 2020). However, the affinity of SR52-KASH to the dynein-dynactin complex and kinesin-1 was several folds weaker than SR48-KASH. The SR53-KASH containing a putative CC1-box motif and the LEWD motif, but no potential Spindly motif, strongly recruited kinesin-1, while weakly bound to dynein-related molecules (**Figure 2-4. D, E**). The shortest construct SR55-KASH containing only a putative CC1-box motif barely bound to either motor. When comparing the relative ratio of dynactin p150^{Glued} over kinesin-1 binding, SR49-KASH and SR48-KASH showed a stronger bias to the dynein-dynactin complex than the previously reported SR52-KASH (**Figure 2-4. F**).

An extended coiled-coil of around 30 nm length flanked by an N-terminal CC1-box and a C-terminal Spindly motif is a shared characteristic among many known dynein activators, which might facilitate docking onto the 37-nm Arp1 filament of the dynactin complex (Urnavicius et al., 2015; Urnavicius et al., 2018). I asked whether SR48-KASH contains such a conserved structural organization for dynein-dynactin recruitment. Protein structure prediction by ColabFold indicated that SR48-KASH contains a rod-shape region formed by spectrin repeats from SR48 to SR53, which is absent in SR52-KASH (**Figure 2-4. G**; Mirdita et al., 2022). The predicted rod-shaped region from SR48 to SR53 is approximately 33 nm long, but it has a low coiled-coil probability as scored by a coiled-coil prediction tool (Ludwiczak et al., 2019). Another puzzling feature is that the putative CC1-box and Spindly motifs are positioned from C-terminus to N-terminus, which is the reversed organization compared to other known dynein activators (**Figure 2-4. H**). And the CC1-box-containing SR55 is separated from the Spindly-motif-containing region by a disordered region containing the LEWD motif, adding more complexity to interpret its mode of function.

Although not in a typical arrangement, we wonder whether these motifs might be involved in binding to the DDB complex. To examine this possibility, I introduced site-specific point

mutations in the conserved amino acids on the putative Spindly (xLF/AxE to AAAAA, referred to as 5A) and CC1-box (AA to VV, referred to as AAVV) motifs (**Figure 2-5. A**). The 5A-AAVV mutant exhibited little or no binding to BicD2, DHC and p150^{Glued} (**Figure 2-5. B, C**), supporting that these motifs are required for binding to the DDB complex. 5A-AAVV mutant showed significantly weaker binding to kinesin-1 than the wildtype SR48-KASH, suggesting that Nesprin-2-DDB binding is not exclusive or competitive with but rather stabilizing Nesprin-2-kinesin-1 binding. I also generated SR48-KASH with point mutations in the kinesin-1-binding LEWD motif (WD to AA, referred to as LEAA) and confirmed that SR48-LEAA failed to bind to kinesin-1 (**Figure 2-5. A-C**). It has previously been reported that the LEAA mutant of SR52-KASH exhibits diminished binding to BicD2 (Gonçalves et al., 2020). In contrast, SR48-LEAA still bound to the DDB complex at a comparable level to control, indicating that SR48-KASH binds to the DDB complex independently of kinesin-1 and the LEWD motif.

It has been reported that BicD2 directly binds to SR52-KASH and recruits both dynein-dynactin and kinesin-1 to Nesprin-2 in migrating cortical neurons (Gonçalves et al., 2020). To further investigate whether similar mechanism can be applied for SR48-KASH, I generated BicD2-knockout HEK293T cell lines using the CRISPR/Cas9 genetic engineering system and performed co-IP assays. In the absence of BicD2, the amount of DHC and p150^{Glued} co-precipitated with SR48-KASH was greatly reduced, suggesting that BicD2 is required for the binding of the dynein-dynactin complex to Nesprin-2 (**Figure 2-5. D, E**). It also showed that the atypical Spindly and CC1 box motifs on SR48-KASH are not sufficient for direct recruitment of dynein and dynactin in the absence of BicD2. In contrast, kinesin-1 was precipitated with SR48-KASH in BicD2-deficient cells at similar levels as the control. These results indicate that BicD2 is indispensable for Nesprin-2-dynein binding, but not for Nesprin-2-kinesin-1 binding.

Taken together, the DDB complex and kinesin-1 are thought to be recruited by Nesprin-2 via different motifs in a non-competitive manner, negating that Nesprin-2 mediates the selective binding of a dominant motor to facilitate unidirectional nuclear transport. It is also unlikely that kinesin-1 assists dynein motor activity by enhancing DDB complex binding to Nesprin-2.

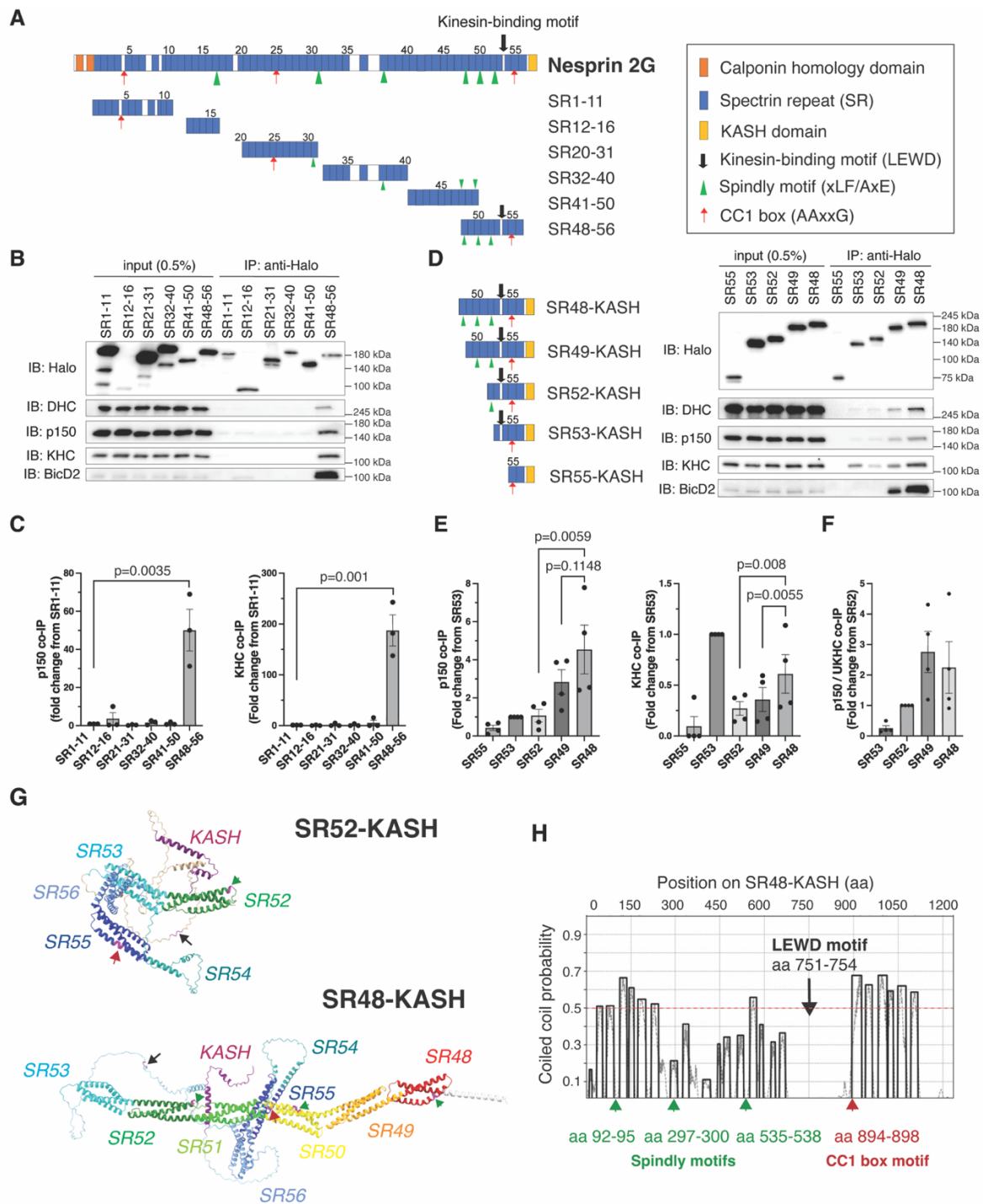


Figure 2-4. Nesprin-2 recruits dynein-dynactin-BicD2 complex and kinesin-1 via separate but spatially close motifs. (A) Schematic of Nesprin-2G and the truncated Nesprin-2 SR fragments highlighting the kinesin-binding motif and the putative dynein-dynactin-binding motifs. **(B, C)** Immunoblotting (B) and quantification (C) of co-IP of HEK293 cell lysate. Cells were transfected with the indicated Halo-tagged Nesprin-2 truncates. $n = 3$ independent experiments. Ratio paired t test was used to compare co-IP results with Ctrl. **(D)** Schematic of Nesprin-2 C-terminal truncates (left) and co-IP results (right) of HEK293 cells transfected with the indicated Halo-tagged Nesprin-2 truncates. **(E, F)** Quantification of co-IP from (D). The quantitative analysis of endogenous p150 and KHC in the pull-down is shown in (E). Ratio paired t test was used to compare co-IP results with

Ctrl. The p150/KHC ratio is shown in (F). $n = 4$ independent experiments. **(G)** Predicted protein structure of SR52-KASH and SR48-KASH by ColabFold. Different colors correspond to different SRs. Black arrows indicate the LEWD motif, green arrowheads indicate the putative Spindly motifs, and red arrows indicate the putative CC1 motifs. **(H)** Predicted coiled coil domains of SR48-KASH by Deepcoil2. Bars show mean $\pm$ SEM.

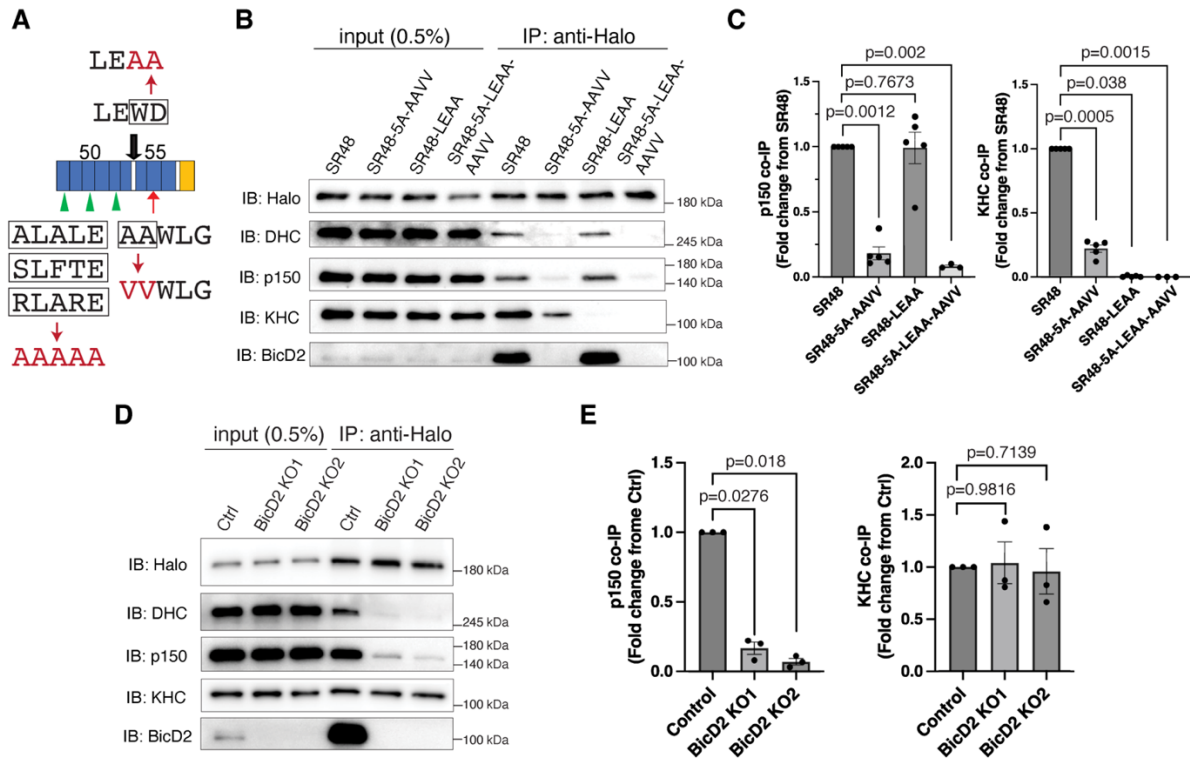


Figure 2-5. Nesprin-2 binds to dynein-dynactin-BicD2 complex and kinesin-1 in a non-competitive manner. **(A)** Schematic indication of the mutation sites on SR48-KASH. In SR48-5A-AAV-KASH construct, all three putative Spindly motifs and one putative CC1-box motif were mutated. In SR48-LEAA-KASH, LEWD motif was mutated. **(B, C)** Immunoblotting **(B)** and quantification **(C)** of co-IP in HEK293 cells transfected with the Halo-tagged wildtype and mutant SR48-KASH. $n = 5$ independent experiments. Ratio paired t test. **(D, E)** Immunoblotting **(D)** and quantification **(E)** of co-IP with Halo-tagged SR48-KASH in control (Ctrl) or BicD2 knockout (KO) HEK293 cell lysates. BicD2 KO1 and KO2 are two independently isolated KO cell lines. $n = 3$ independent experiments. Ratio paired t test was used to compare with Ctrl. Bars show mean $\pm$ SEM.

2.2.2 Nesprin-2-deficient CGCs showed delayed nuclear migration during cerebellum development

To further investigate whether Nesprin-2 is involved in nuclear migration during cerebellum development, I established a Nesprin-2-deficient mutant mouse line by the improved Genome-editing via Ovidutal Nucleic Acids Delivery (i-GONAD) method. The i-GONAD method is based on CRISPR/Cas9-mediated genome editing and I obtained a mouse line with a 16-bp deletion in the 108th exon on *Nesprin-2/Syne2* gene, which results in frameshift and a potential pre-mature stop codon at 30-bp downstream. Sequencing and genotyping were performed to confirm the mutation (**Figure 2-6. A, B**). The mice with such deletion are viable and fertile without major behavioral defects, but they seem to have a shorter life expectancy (data not shown). Western blot with lysate from mouse cerebella showed drastically reduced levels of full-length Nesprin-2G while the protein bands of other Nesprin-2 isoforms were still present (**Figure 2-6. C**). Therefore, this mouse line is addressed as a Nesprin-2 mutant mouse line because it is not a complete knockout. Nevertheless, immunostaining of mouse cerebellum tissue sections as well as primary cultures of CGCs from this mutant mouse line showed only traces of diffusive Nesprin-2 signals in the cytoplasm while their wildtype littermate showed clear Nesprin-2 signal decorating the nuclear envelope (**Figure 2-6. D, E**). Although the detailed protein product from the mutated *Nesprin-2* gene is unknown, it is assumed that the nuclear-locating Nesprin-2 is depleted, producing a phenotypic null animal model for analysis of neuronal nuclear translocation.

In Nesprin-2 mutant mice, the gross anatomy of the brain appears normal, but the size of the cerebella are significantly smaller than wildtype mice at postnatal day 9 and 12 (P9 and P12) when CGC migration is active (**Figure 2-7. A, B**). The cerebellar sizes become comparable between mutant and wildtype after cortex formation is completed by P30. During normal development, CGCs are generated by cell division within the outer external granule layer (oEGL), followed by tangential migration and radial migration from the inner external granule layer (iEGL), through the molecular layer (ML) toward the inner granule layer (IGL). At P9 and P12, Nesprin-2 mutant mice have a much thicker iEGL, which is populated with p27-Kip1-positive post-mitotic cells (**Figure 2-7. C-E**). The thickness of oEGL with Ki-67-positive proliferative cells is not changed. These results indicate that, while CGC proliferation is unaffected, CGC migration from the EGL to IGL is delayed in the absence of functional Nesprin-2.

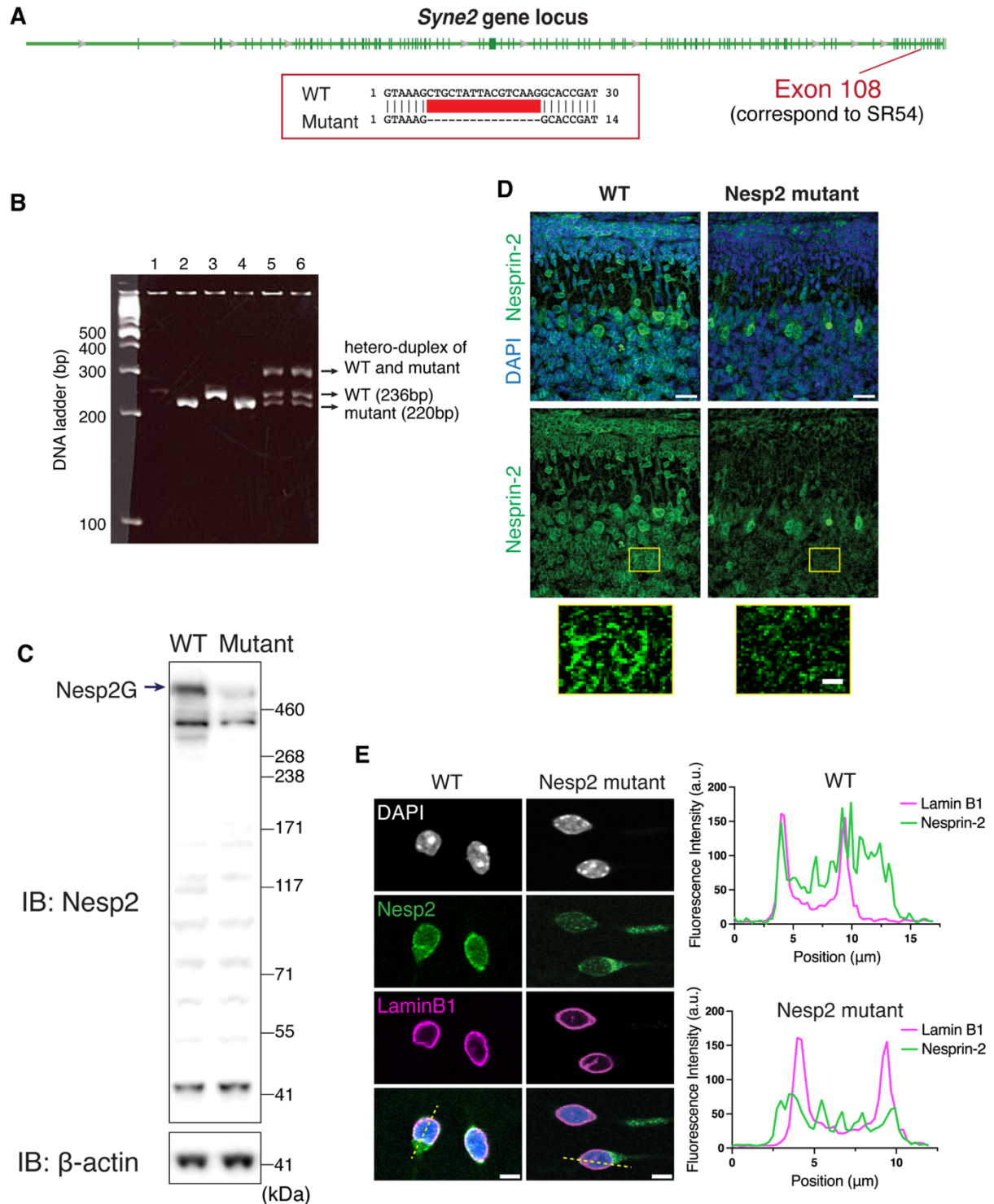


Figure 2-6. Validation of Nesprin-2 mutant mouse line. (A) Annotation of *Syne2* gene (NM_001005510.2) from NCBI RefSeq. The deletion site and the mutated sequences in the mutant mouse line are indicated. Exons are shown as green bars and transcription direction is indicated by gray arrows. (B) An example of genotyping results. PCR products from heterozygous and homozygous wildtype (WT) and Nesprin-2 mutant mice were distinguished by agarose gel electrophoresis. Numbers indicate different mice. (C) Western blot of the lysates from P9 WT and Nesprin-2 mutant cerebelli. Full-length Nesprin-2G corresponds to a band at ~800 kDa. (D) Immunofluorescence staining of Nesprin-2 in the cerebella from P9

WT and Nesprin2 mutant mice. The zoom-in views of the selected regions are shown. (E) Immunocytochemistry staining of Nesprin-2 and Lamin B1 in CGCs from WT and Nesprin-2 mutant mice. The fluorescence intensity is plotted along the dashed yellow lines. Scale bars, 20 μm in (D) upper panels, 5 μm in (D) lower panels and (E).

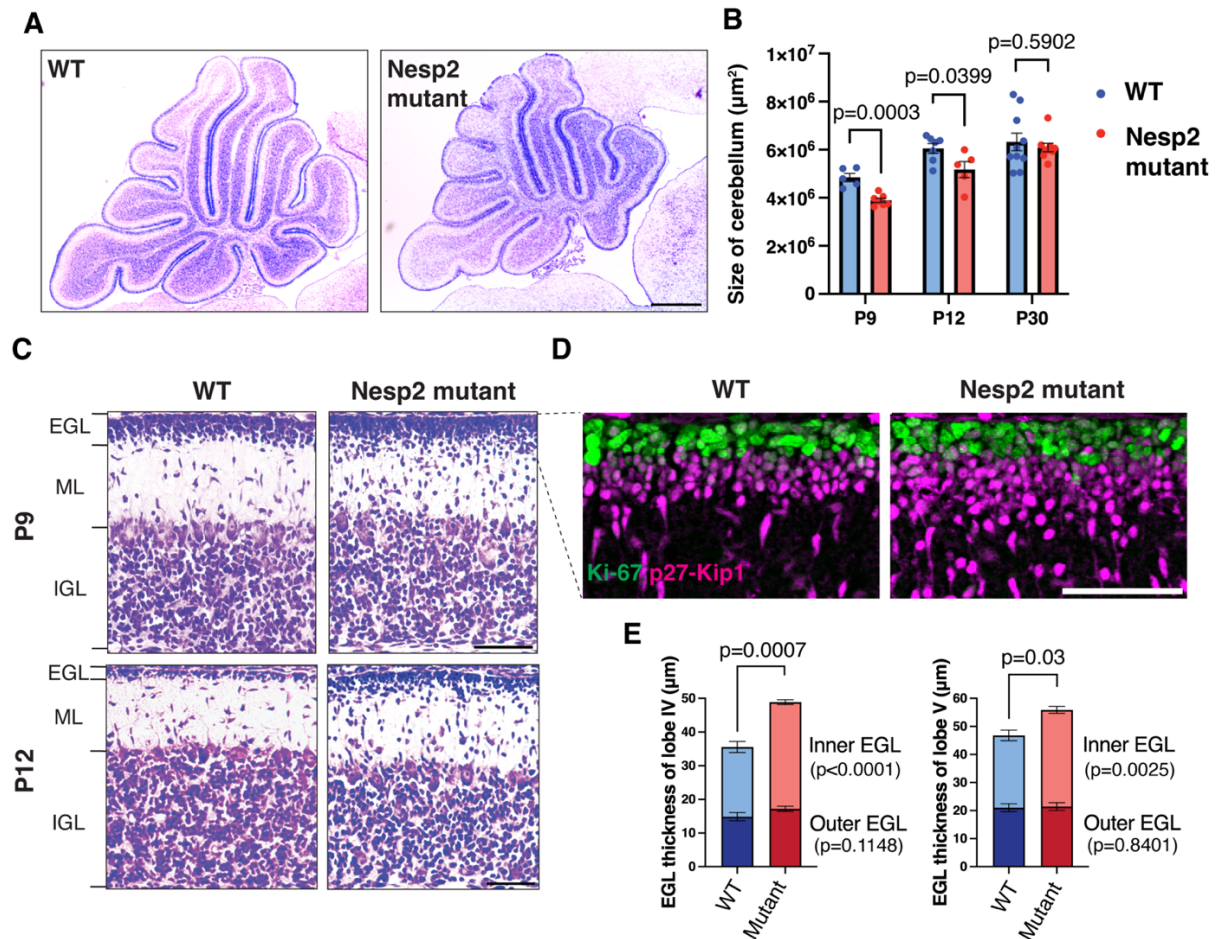


Figure 2-7. Mislocalization of CGC nuclei in Nesprin2 mutant mice during cerebellum development. (A) Nissl staining of midsagittal cryosections of P9 cerebella from WT and Nesprin-2 mutant mice. (B) Area quantification of midsagittal sections of P9, P12 and P30 samples. $n = 5, 7$ and 10 mice (WT), $n = 7, 5$ and 9 mice (Nesprin-2 mutant) from $3 \sim 4$ independent experiments. Unpaired t test. (C) Enlarged views of the mid region of cerebellum lobe IV from WT and Nesprin-2 mutant mice at P9 and P12. EGL, ML and IGL can be distinguished by visual inspection. (D) Immunofluorescence staining of Ki-67 (green) and p27-Kip1 (magenta) in the EGL from WT and Nesprin-2 mutant mice. (E) Thickness quantification of the outer and inner EGL of cerebellar lobe IV (left) and lobe V (right). $n = 5$ (WT) and 7 (Nesprin-2 mutant) mice from 3 independent experiments. Unpaired t test was used to compare WT and Nesprin-2 mutant. Bars show mean $\pm$ SEM. Scale bars, $500 \mu\text{m}$ in (A); $50 \mu\text{m}$ in (C, D).

2.2.3 Nesprin-2 SR48-KASH rescues impaired nuclear migration by binding with both dynein and kinesin-1

I then analyzed the nuclear migration dynamics of CGCs from Nesprin-2 mutant mice in reaggregate culture. When observing the cell morphology, Nesprin-2 mutant CGCs have a longer distance between the nucleus and the centrosome, which is an indicator for nucleus-centrosome coupling (**Figure 2-8. A**). Time-lapse imaging revealed that mutant CGCs have a slower nuclear migration speed *in vitro* (mean displacement 7.46 μm per hour) than wildtype cells (mean displacement 12.91 μm per hour) (**Figure 2-8. B, C**). The rotation movements of the nucleus also decreased in Nesprin-2 mutant CGCs, further indicating disconnection between the nucleus and microtubule motors (**Figure 2-8. D**; Wu et al., 2018).

I next performed rescue experiments by transfecting the cultured CGCs from mutant mice with a recombinant construct of mNeonGreen (mNG) fused with Nesprin-2 SR48-KASH. The mNG signal was localized on the nuclear envelope, indicating successful integration of exogenous SR48-KASH in the nuclear membrane (**Figure 2-8. E**). The displacement of nuclei from Nesprin-2 mutant CGCs resumed to the level of wildtype CGCs (**Figure 2-8. C, E**). Although a large portion of Nesprin-2G is missing in SR48-KASH construct, the rescue of nuclear migration was achieved, which is consistent with a previous report by Gonçalves et al. (2020) that actin-binding activity of the N-terminal CH domains on Nesprin-2G is not required for neuronal migration. In contrast, transfection of the non-dynein-binding 5A-AAVV mutant or the non-kinesin-binding LEAA mutant of SR48-KASH failed to recover nuclear movements (**Figure 2-8. C, E**). It suggests that binding with both dynein complex and kinesin-1 is required for Nesprin-2 to exert nuclear transport function. Taken together with the results from Chapter 1, it is conceivable that recruitment of both dynein and kinesin-1 to Nesprin-2 is essential for forward nuclear movements in migrating CGCs.

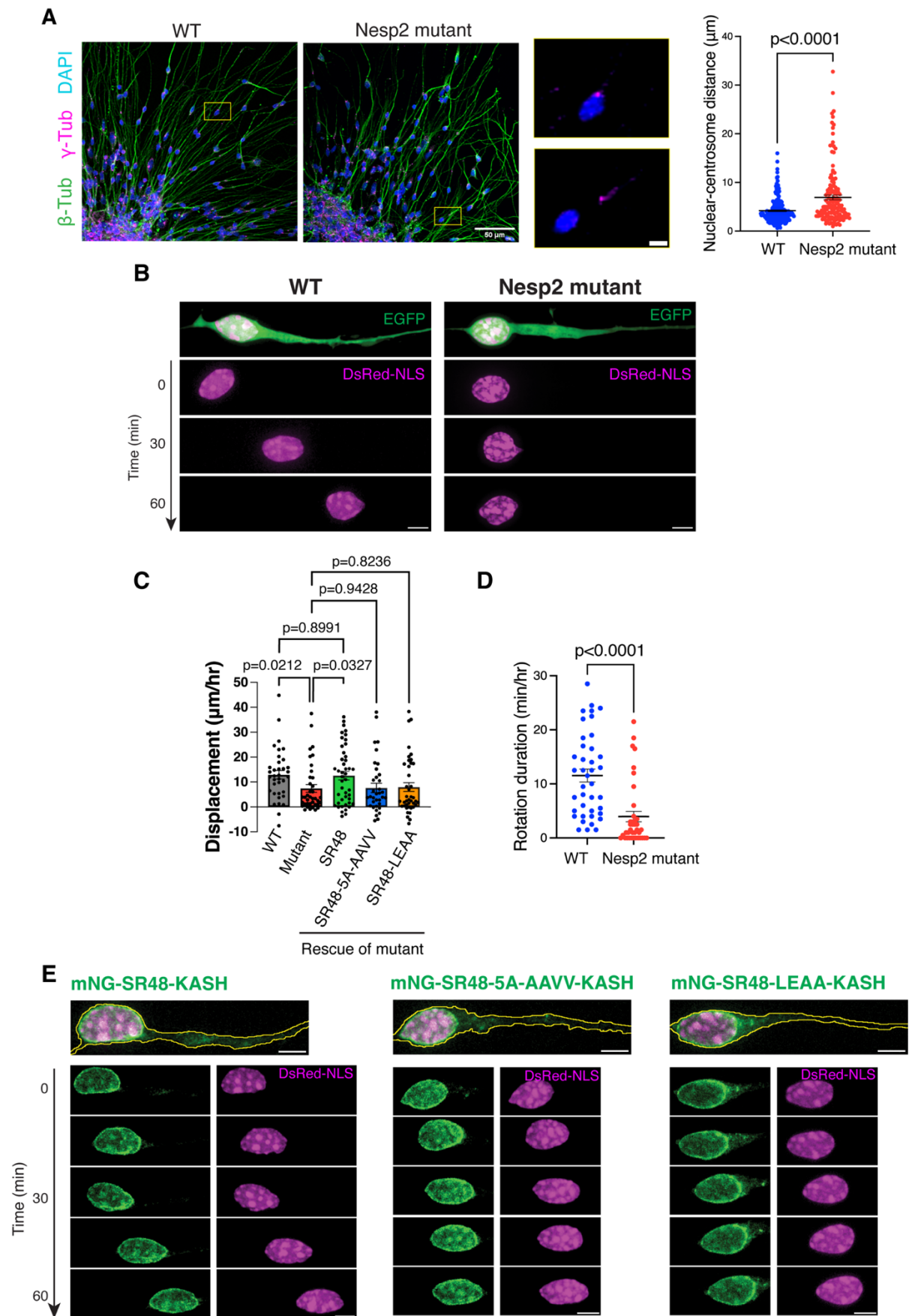


Figure 2-8. Binding to both dynein and kinesin-1 is necessary for Nesprin-2 to drive nuclear migration in CGCs. (A) Immunocytochemistry staining (*left*) and quantitative

analysis (*right*) of the nucleo-centrosome distance in CGC reaggregate cultures. CGCs from WT and Nesprin-2 mutant mice were stained with β -tubulin (*green*) and γ -tubulin (*magenta*). The zoom-in views of the selected regions are shown. $n = 205$ (WT) and 148 (mutant) cells. Unpaired t test with Welch's correction. **(B)** Time-lapse sequences of representative CGCs in reaggregate cultures. CGCs from WT and Nesprin-2 mutant mice were transfected with EGFP (*green*) and DsRed-NLS (*magenta*). **(C)** Quantitative analysis of nuclear net displacement. $n = 36$ (WT), 38 (Nesp2 mutant), 45 (Nesprin-2 mutant cells transfected with SR48-KASH), 36 (Nesprin-2 mutant cells transfected with SR48-5A-AAVV-KASH) and 41 (Nesprin-2 mutant cells transfected with SR48-LEAA-KASH) cells from 4 ~ 5 independent experiments per group. Unpaired t test. **(D)** Quantification of nuclear rotation in reaggregate cultures of CGCs from WT and Nesprin-2 mutant mice. During 1-hour time-lapse imaging, duration of the nuclear rotation over 10° was counted. $n = 40$ (WT) and 38 (mutant) cells from 4 independent experiments. Unpaired t test. **(E)** Time-lapse sequences of representative CGCs from Nesprin-2 mutant mice. CGCs were transfected with DsRed-NLS (*magenta*) together with mNG-SR48-KASH, mNG-SR48-5A-AAVV-KASH or SR48-LEAA-KASH (*green*). Bars show mean $\pm$ SEM. Scale bars, 50 μm in (A) left panels, 5 μm in (A) middle panels and (B, E).

2.2.4 Nesprin-2 generates persistent cargo movements bidirectionally along microtubules

To investigate how Nesprin-2 regulates the activities of opposing microtubule motors, I adopted a drug-inducible cargo trafficking assay to examine the intracellular transport dynamics of Nesprin-2-dynein-kinesin-1 complex (Kapitein et al., 2010). This assay utilizes the heterodimerization of FKBP (FK506 binding protein) and FRB (FKBP-rapamycin binding domain) proteins upon treatment of a cell-permeable drug, rapalog. By co-transfection of a peroxisome-targeting construct PEX-GFP-FKBP and a FRB construct conjugated with specific motors or motor adaptors in cultured cells, rapalog induces docking of motor molecules onto peroxisomes, which are usually non-motile, and then peroxisome movements are initiated upon association with motors or motor adaptors (**Figure 2-9. A**). This assay provides a well-controlled method to characterize intracellular cargo transport generated by designated molecules. I performed this cargo trafficking assay in cell lines with relatively simple microtubule organization as well as in cultured CGCs with a more complex microtubule arrangement.

Firstly, I validated this assay in COS7 cells. Microtubules in COS7 cells are organized as radial arrays emanating from the microtubule organizing center (MTOC) to cell periphery, which could be demonstrated by the inside-out movements of the microtubule-plus-end

protein EB3 (**Figure 2-9. B**). I used the constitutively active form of Kif5B (amino acids 1-807) to be conjugated with FRB. When Kif5B was recruited onto peroxisomes, they quickly dispersed from peri-nuclear region to cell periphery, where microtubule plus ends are located (**Figure 2-9. C**). On the contrary, when the dynein activator, BicD2 (amino acids 1-509) was coupled onto peroxisomes, they rapidly gathered at the MTOC, where microtubule minus ends are located (**Figure 2-9. C**). SR48ΔKASH was then used to recapitulate microtubule-motor-driven transport generated by Nesprin-2. As SR48ΔKASH binds to both dynein complex and kinesin-1, I hypothesized that it would generate bidirectional movements by combining the minus-end-directed and the plus-end-directed transport by opposing motors. Upon rapalog addition, SR48ΔKASH indeed moved peroxisomes both toward and away from the cell centre. (**Figure 2-9. C**). I noted that, while Kif5B or BicD2 executed rapid and unidirectional transport of cargoes, SR48ΔKASH kept the cargoes moving bidirectionally between the MTOC and cell periphery, which could be reflected by plotting the positions of GFP-labelled peroxisomes at different time points (**Figure 2-9. D**). I further analyzed the number of actively moving peroxisomes over time, and found that Kif5B- or BicD2-driven cargo movements peaked shortly after drug treatment followed by declined motility when most peroxisomes reached and paused at the microtubule ends (**Figure 2-9. E**). In contrast, SR48-mediated cargo movements remained active throughout the 1-hour observation period. This persistency in cargo motility generated by Nesprin-2 might have important implications in neuronal nuclear translocation.

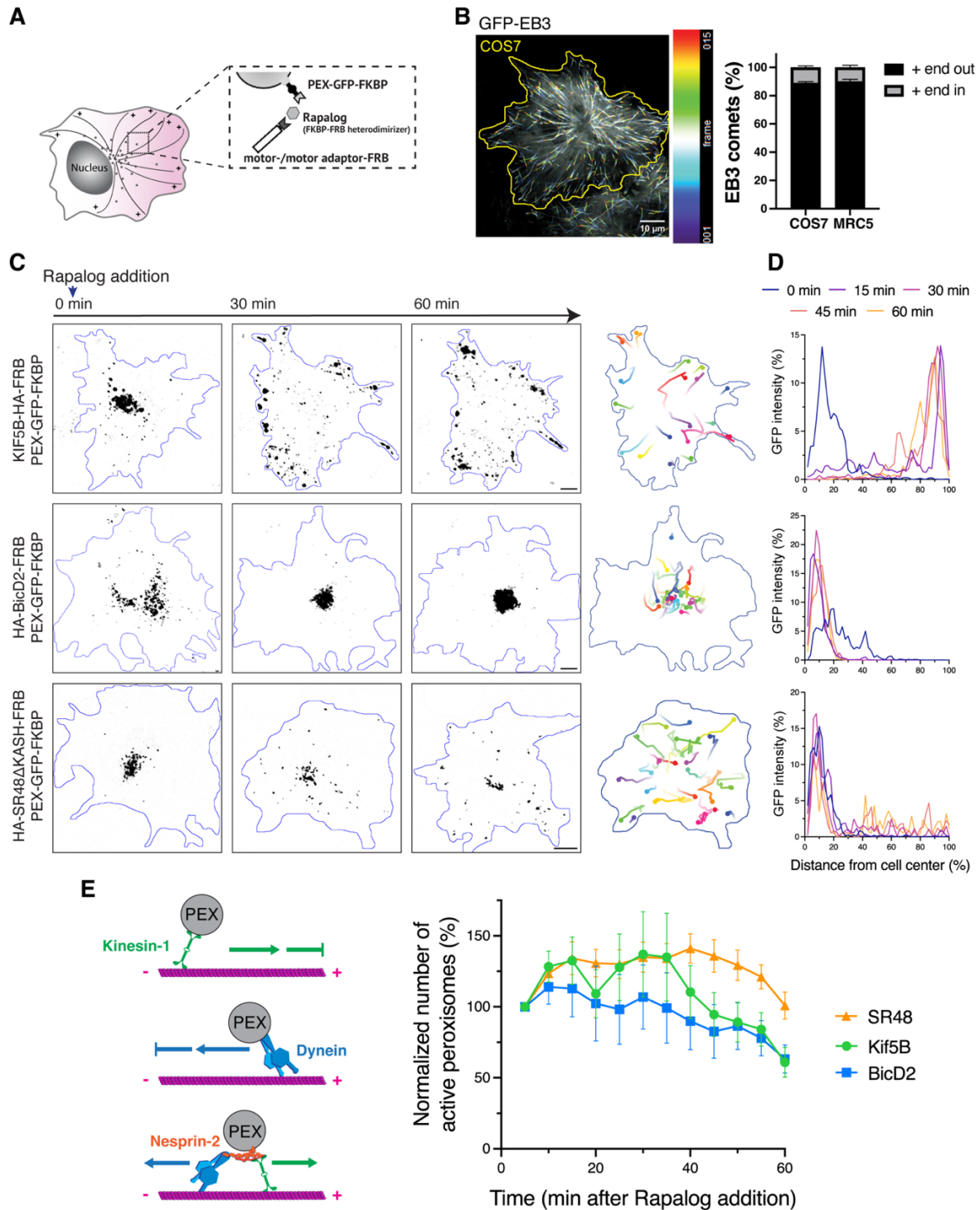


Figure 2-9. Nesprin-2 generates active bidirectional cargo transport over an extended duration. (A) Schematic of the drug-inducible peroxisome trafficking assay. Adapted from Kapitein et al. (2010). (B) *Left*, temporal color-coded time-lapse images of a COS7 cell transfected with GFP-EB3. Images were taken at 1-sec intervals. Projection of 15 frames are shown. *Right*, analysis of EB3 comet movements by KymoButler. In both COS7 and MRC5 cells, around 90% of EB3 comets are moving toward cell periphery. $n = 15$ (COS7) and 11 (MRC5) cells from 3 independent experiments, and over 100 trajectories were analyzed per cell. (C) Inducible peroxisome trafficking assay in COS7 cells transfected with PEX-GFP-FKBP together with KIF5B-HA-FRB (*top*), HA-BicD2-FRB (*middle*), or HA-SR48ΔKASH-

FRB (*bottom*). Images at 0, 30 and 60 min after rapalog treatment are shown. GFP signals are shown in black and cell contours are outlined in blue. *Right*, Representative trajectories of GFP-labelled peroxisomes are shown with lines by different colors, with time denoted by color gradient and ending points denoted by filled circles. **(D)** Distribution of GFP-labelled peroxisomes along the distance from cell center to cell periphery at 0, 15, 30, 45 and 60 min after rapalog treatment. Kif5B- or BicD2-expressing cells show rapid PEX-GFP displacement toward the cell edge or cell center, respectively. Nesprin-2-SR48-expressing cells show persistent fluctuation of GFP distribution, indicating continuous bidirectional cargo transport. **(E)** *Left*, schematic of peroxisome movements driven by kinesin-1, dynein/BicD2 and Nesprin-2. Flat-headed arrows indicate termination of transport. *Right*, quantification of active peroxisomes in respective time windows. The number of peroxisomes that moved for 3 μm or more were counted. The values are normalized to the number of moving peroxisomes in the first time window (0 ~ 5 min). $n = 9$ (Kif5B), 16 (BicD2) and 16 (SR48) cells from 3 independent experiments per group. Data points and error bars show mean $\pm$ SEM. Scale bars, 10 μm in (B, C).

2.2.5 Nesprin-2 mediates active cargo transport when bound with opposing motors

I performed more in-depth analysis on peroxisome movements by dual-color time-lapse imaging at a higher temporal-spatial resolution. Together with GFP-labelled peroxisomes, intracellular microtubules were labelled by transfection of mCherry- α -tubulin (**Figure 2-10. A**). MRC5 cells were used for better visualization due to its flat morphology and sparsely-located microtubules, which have a plus-end-out microtubule arrangement like COS7 cells (**Figure 2-9. B**). I performed imaging at 10 Hz frequency to trace movements of single peroxisomes along microtubule tracks. Kif5B and BicD2 generated uni-directional cargo movements, but toward opposite directions along microtubules (**Figure 2-10. B, C**). DHC construct was used to allow characterization of dynein-driven transport without BicD2-dependent activation, which also showed minus-end-directed cargo movements (**Figure 2-10. D**). Consistent with the observation in COS7 cells, SR48 Δ KASH-coupled peroxisomes presented a mixture of bidirectional movements (**Figure 2-10. E**). Kymographs were generated by plotting the positions of a peroxisome along a single microtubule at each time frame, which were used for quantifying cargo kinetics with a semi-automated tool KymoAnalyzer (Neumann et al., 2017). I extracted the processive moving segments from the kymographs for velocity and run length analysis where peroxisomes moved at constant speed for over 1- μm travel distance. Previous studies on intracellular cargo transport and single-molecule imaging demonstrated that kinesin-1 moves at velocities of 0.5 ~ 0.8 $\mu\text{m}/\text{s}$ while

DDB complex exhibits a broader distribution of velocities ranging from 0.9 ~ 2 $\mu\text{m/s}$ (Thorn et al., 2000; Seitz and Surrey, 2006; Nath et al., 2007; Ross et al., 2008; Lu et al., 2009; McKenney et al., 2014; Schlager et al., 2014; Elshenawy et al., 2019; Htet et al., 2020; Serra-Marques et al., 2020; Gicking et al., 2022). The velocities measured from my data were within the range of the previously reported speed. For plus-end directed transport, Kif5B moved the peroxisomes at an average speed of 0.58 $\mu\text{m/s}$ (**Figure 2-10. F**). For minus-end directed transport, DHC moved at slower velocities (0.70 $\mu\text{m/s}$ on average) than BicD2-FRB (average 1.05 $\mu\text{m/s}$ on average), supporting that BicD2 enhances the stability of dynein-dynactin complex and promotes its motor activity (McKenney et al. 2014; Schlager et al. 2014; Chowdhury et al. 2015). Nesprin-2 SR48 Δ KASH generated bidirectional movements at around 1 $\mu\text{m/s}$ toward both directions (0.93 $\mu\text{m/s}$ toward plus ends and 0.96 $\mu\text{m/s}$ toward minus ends), suggesting that SR48 Δ KASH recruited both DDB complex and kinesin-1 for cargo transport. Intriguingly, association with SR48 Δ KASH enhanced the plus-end-directed cargo transport speed by kinesin-1, while the minus-end-directed transport by SR48 Δ KASH was comparable to BicD2. The average run length of SR48 Δ KASH-mediated transport to minus- and plus-ends were indistinguishable (2.48 μm toward plus ends and 2.47 μm toward minus ends), while the values were slightly lower than BicD2- or Kif5B-driven transport, respectively (**Figure 2-10. G**). SR48 Δ KASH also showed a slightly higher frequency of minus-end-directed than plus-end-directed transport ($60 \pm 10\%$ toward minus ends) (**Figure 2-10. H**), suggesting a biased movement towards the microtubule minus ends.

I next examined the SR48 mutants that are depleted of kinesin-1- or DDB-binding in the peroxisome transport assay. SR48-LEAA mutant, which binds to DDB complex but not to kinesin-1, exhibited very limited transport activities to both ends of microtubules (**Figure 2-11. A, B**), suggesting that kinesin-1 binding enhances rather than competes with dynein-mediated transport. On the contrary, SR48-5A-AAVV mutant, which is unable to bind to dynein complex, moved peroxisomes toward the cell periphery, but less efficiently than Kif5B (**Figure 2-11. A, B**). These results suggest that Nesprin-2-mediated cargo transport toward microtubule minus ends requires both dynein and kinesin-1 binding, while the plus-end directed transport requires only kinesin-1 binding.

The cargo dynamics generated by SR48-LEAA and SR48-5A-AAVV mutants were also analyzed based on kymographs derived from dual-color time-lapse imaging. Although the mutants showed much less active peroxisome movements overall, I recorded occasional processive transport when imaging at sub-second time intervals. The non-kinesin-bound

LEAA mutant exhibited a strong bias to minus-end transport, while the non-dynein-bound 5A-AAVV mutant exhibited a strong bias to plus-end transport (**Figure 2-11. C, D, G**). Both mutants showed slower velocities than wildtype SR48 Δ KASH in either minus-end- or plus-end-directed transport, respectively (0.72 $\mu\text{m/s}$ for minus-end transport by SR48-LEAA and 0.51 $\mu\text{m/s}$ for plus-end transport by SR48-5A-AAVV; **Figure 2-11. E**), indicating less efficient transport of the working motor in the absence of its opposing motor. The mutants did not change the run lengths of cargo transport (**Figure 2-11. F**). From these results, I concluded that Nesprin-2 SR48 Δ KASH enhances transport velocities by kinesin-1 and dynein in the presence of both motors, but single-motor-bound mutants are incapable of fully activating the associated motors.

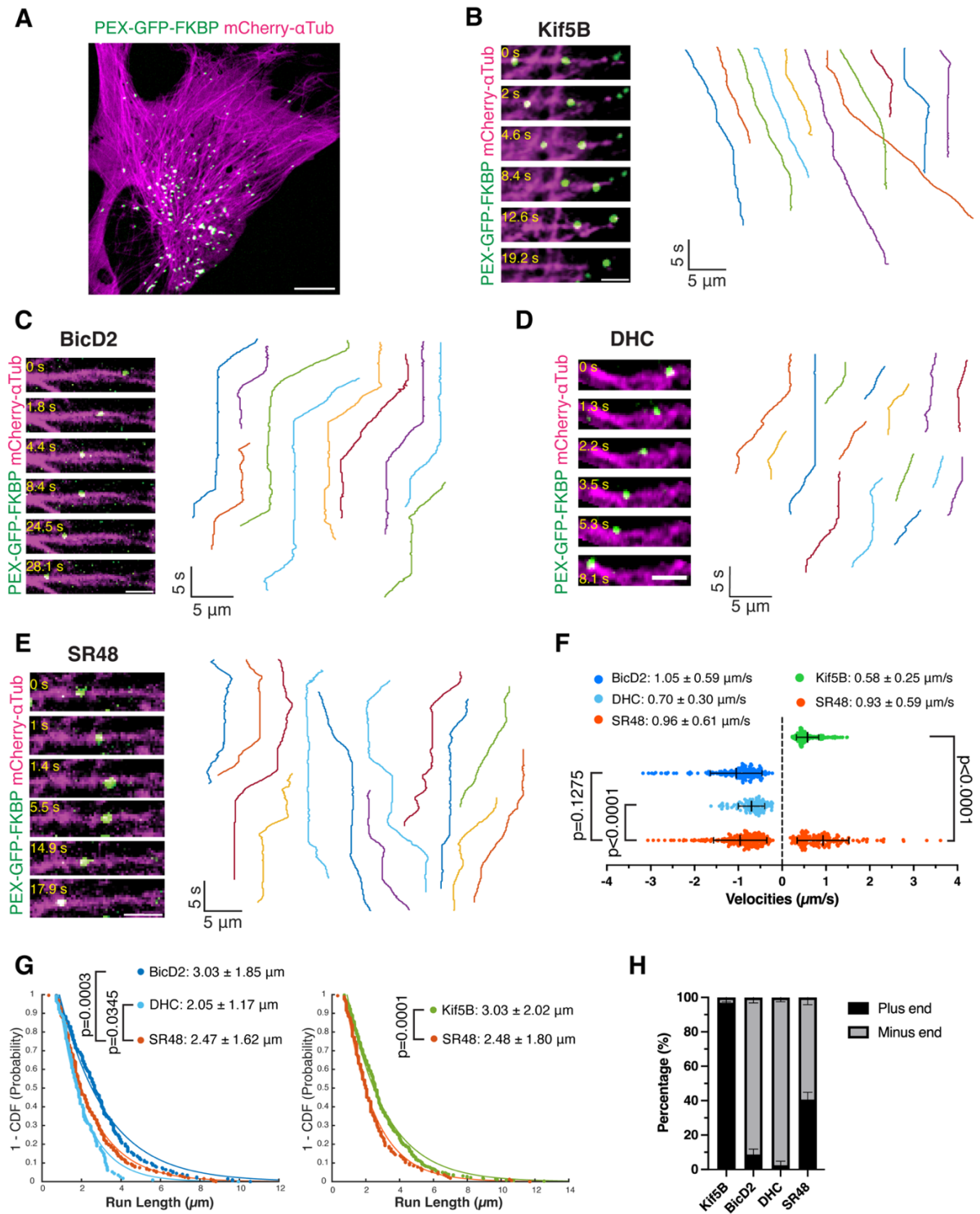


Figure 2-10. Nesprin-2 acquires a novel profile of cargo transport kinetics. (A) Representative image of an MRC5 cells transfected with PEX-GFP-FKBP (green), mCherry- α -tubulin (magenta) and FRB constructs conjugated with motors or motor adaptor. (B-E) Representative time-lapse sequences (left) and kymographs (right) of rapalog-induced peroxisome transport by Kif5B (B), BicD2 (C), DHC (D) and Nesprin-2 SR48 (E). Images were taken at 0.1-sec intervals in MRC5 cells with microtubules (MTs) oriented from minus (left) to plus (right) ends. Kymographs from different experiments are shown by different colors. Position of peroxisomes are represented on the horizontal axis from left to right, while

time is represented on the perpendicular axis from top to bottom. The leftmost kymographs (*blue*) in each panel corresponds to the peroxisomes shown in the time-lapse sequences. **(F)** Velocity distribution of peroxisomes transported by Kif5B (*green*), BicD2 (*blue*), DHC (*cyan*), and Nesp2-SR48 (*orange*). Velocities from processive transport segments with distance over 1 μm are included. Positive velocities indicate movement toward MT plus ends and negative velocities indicate movement toward MT minus ends. Unpaired t test with Welch's correction. **(G)** Inverse cumulative distribution functions (CDF) of run length toward MT minus end (*left*) and plus end (*right*). Lines show the fitted single exponential decay curves and data points show the actual values. Mann Whitney test. **(H)** Percentages of runs toward MT plus and minus ends. $n = 343$ (Kif5B), 188 (BicD2), 103 (DHC), 147 (SR48 to MT plus end), and 226 (SR48 to MT minus end) trajectory segments in more than 25 cells from 3 ~ 5 independent experiments per group in (F-H). Bars show mean $\pm$ SEM. Numerical values are mean $\pm$ SD. Scale bars, 10 μm in (A); 2 μm in (B-E).

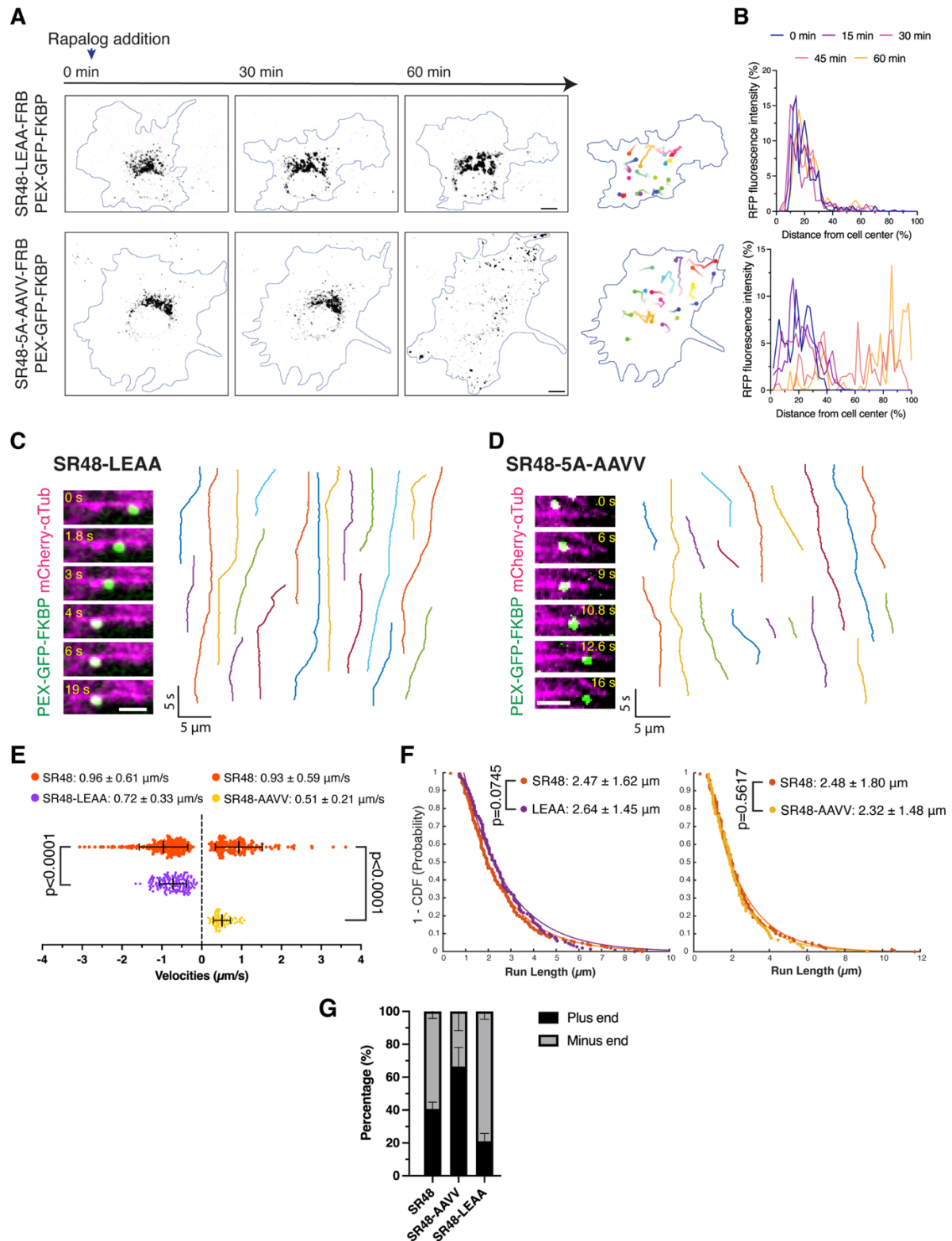


Figure 2-11. Nesprin-2 mutants exhibit reduced transport activities. (A) Rapalog-induced peroxisome transport by Nesp2-SR48 Δ KASH-LEAA (SR48-LEAA; *top*) and Nesp2-SR48 Δ KASH-5A-AAVV (SR48-5A-AAVV; *bottom*). Images at 0, 30 and 60 min after rapalog treatment are shown. GFP signals are shown in black and cell contours are outlined in blue. *Right*, Representative trajectories of GFP-labelled peroxisomes are shown with lines by different colors, with time denoted by color gradient and ending points denoted by filled

circles. **(B)** Distribution of GFP-labelled peroxisomes along the distance from the cell center to the cell periphery at 0, 15, 30, 45 and 60 min after rapalog treatment. SR48-LEAA showed limited peroxisome movement, while SR48-5A-AAVV triggered slow migration toward the cell periphery. **(C, D)** Representative time-lapse sequences (*left*) and kymographs (*right*) of rapalog-induced peroxisome transport by SR48-LEAA and SR48-5A-AAVV. Images were taken at 0.1-sec intervals in MRC5 cells with MTs oriented from minus (*left*) to plus (*right*) ends. Kymographs from different experiments are shown by different colors. Position of peroxisomes are represented on the horizontal axis from left to right, while time is represented on the perpendicular axis from top to bottom. The leftmost kymographs (*blue*) in each panel corresponds to the peroxisomes shown in the time-lapse sequences. **(E)** Velocity distribution of peroxisomes transported by SR48 (*orange*), SR48-LEAA (*purple*) and SR48-AAVV (*yellow*). Velocities from processive transport segments with distance over 1 μm are included. Positive velocities indicate movement toward MT plus ends and negative velocities indicate movement toward MT minus ends. Unpaired t test with Welch's correction. **(F)** Inverse cumulative distribution functions (CDF) of run length toward MT minus end (*left*) and plus end (*right*). Lines show the fitted single exponential decay curves and data points show the actual values. Mann Whitney test. **(G)** Percentages of runs toward MT plus and minus ends. n=147 (SR48 to MT plus end), 226 (SR48 to MT minus end), 143 (SR48LEAA) and 82 (SR48-AAVV) trajectory segments in more than 25 cells from 3 ~ 5 independent experiments per group. Bars show mean $\pm$ SEM. Numerical values are mean $\pm$ SD. Scale bars, 10 μm in (A); 2 μm in (C-D).

2.2.6 Nesprin-2-mediated cargo transport is not subject to size effects

Cargo transport by Nesprin-2 SR48 Δ KASH was only slightly biased towards the microtubule minus-end direction, but this alone cannot account for the strongly forward-biased movements of the nucleus in migrating neurons. I suspected that the enlarged size of a nucleus (with a radius around 4 μm) compared to a peroxisome (with a radius around 0.25 μm) might influence the directional preference between microtubule-based minus-end- and plus-end-directed movements. It has been reported that multiple dynein molecules linked to the same cargo cooperate better than multiple kinesin molecules, generating larger forces and more persistent movements (Rai et al., 2013). As the large nucleus might recruit a large number of Nesprin-2 molecules which might mediate cooperative function of associated motors, I investigated whether increasing the cargo size would change the directional preference of Nesprin-2-mediated transport.

This possibility was tested by increasing the size of peroxisomes with mVenus-SKL (amino acids Ser-Lys-Leu) overexpression. SKL sequence encodes a short tag at the C-terminus for protein to be imported into peroxisome lumen, hence increases the physical size of peroxisomes (**Figure 2-12. A**; Efremov et al., 2014). I performed cargo transport assay using

COS7 cells transfected with mVenus-SKL in which the peroxisome size was increased by 1.6 folds (**Figure 2-12. B**). The overall mobility of peroxisomes decreased; however, I did not see an increased bias of Nesprin-2-mediated transport toward the cell center (**Figure 2-12. C**).

As another strategy to increase cargo size, I tried overexpression of PEX-mCherry-GM130^{C-ter} (C-terminus of Golgi matrix protein 130), which artificially ties peroxisomes in a row and increased its size by 3.8 folds (**Figure 2-12. A, B**; Fukumitsu et al., 2015). SR48ΔKASH moved these large peroxisome clusters much less efficiently than moving normal peroxisomes, but little effect on the bias of trafficking direction could be observed (**Figure 2-12. C**). These results negate that multiple Nesprin-2 molecules mediate cooperative function of motors bound to each Nesprin molecule.

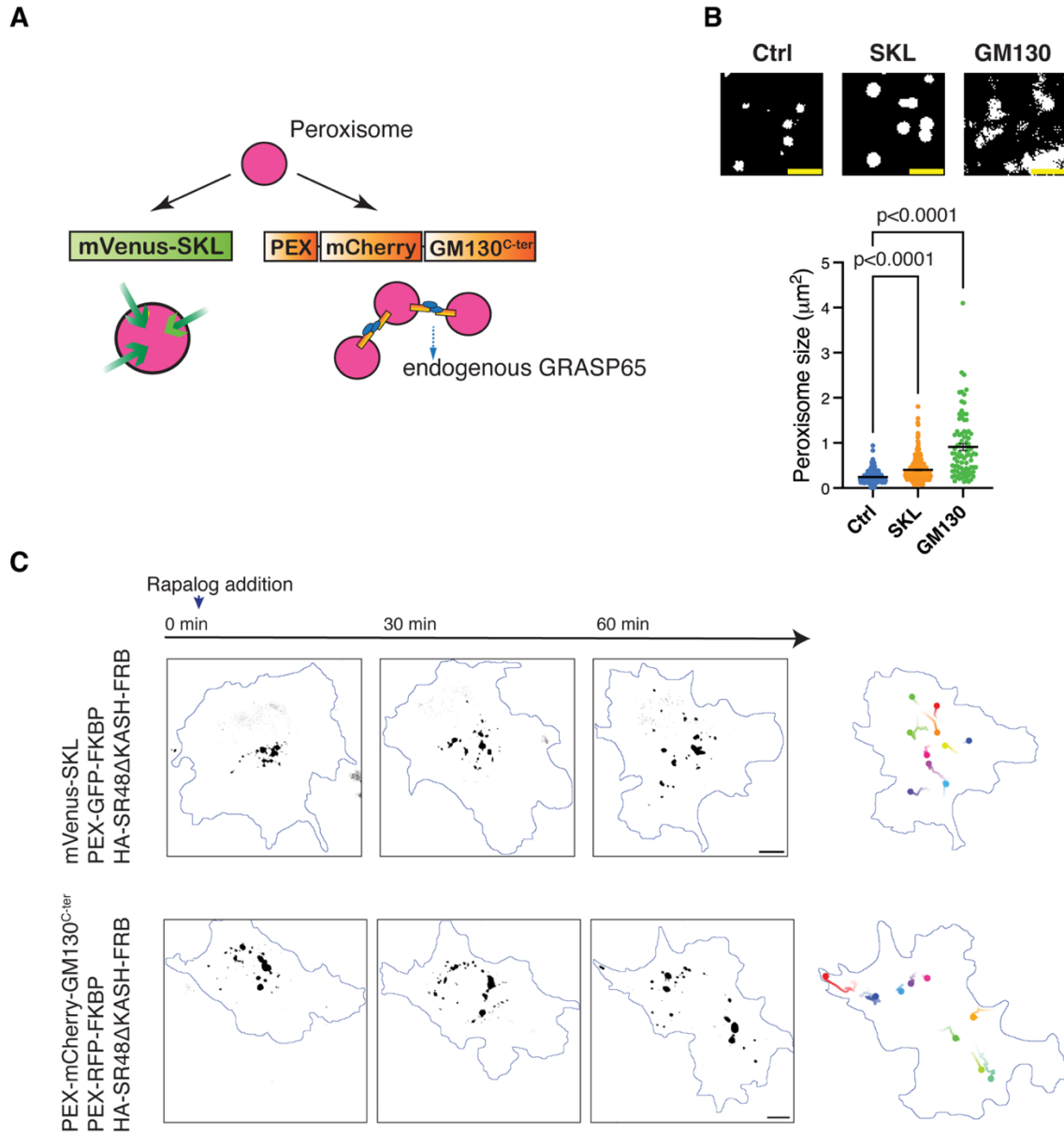


Figure 2-12. Increased size of peroxisomes does not promote dynein-dominating movements by Nesprin-2. (A) Schematic of strategies for increasing peroxisome size. SKL-tagged proteins are imported into the peroxisomes and increase the peroxisome size. GM130^{C-ter} cross-links multiple peroxisomes through homo-oligomerization of intracellular GRASP65 protein. **(B)** Quantification of peroxisome size. COS7 cells were transfected with PEX-GFP-FKBP only (Ctrl), PEX-GFP-FKBP and mVenus-SKL (SKL), or PEX-RFP-FKBP and PEX-mCherry-GM130^{C-ter} (GM130). The area of single peroxisomes was measured. $n = 284$ (Ctrl), 287 (SKL) and 87 (GM130) peroxisomes from $4 \sim 6$ cells per group. Unpaired t test with Welch's correction. Bars show mean $\pm$ SEM. **(C)** Rapalog-induced peroxisome transport by Nesp2-SR48ΔKASH in COS7 cells expressing mVenus-SKL or PEX-mCherry-GM130^{C-ter}. Representative trajectories are shown with lines by different colors. Scale bars, $2 \mu\text{m}$ in (B); $10 \mu\text{m}$ in (C).

2.2.7 Nesprin-2 generates persistent cargo transport bidirectionally in migrating CGCs

To further understand Nesprin-2-driven cargo motilities in migrating neurons, I performed the peroxisome assay in reaggregate cultures of CGCs. In migrating neurons, microtubules are extending from the MTOC near the cell soma and are arranged uniformly with plus ends pointing toward the distal tips in the leading and the trailing processes. I transfected CGCs with a PEX-mCherry-FKBP construct and an FRB construct fused with Kif5B (amino acids 1-807), BicD2 (amino acids 1-509) or SR48 Δ KASH. Rapalog treatment induced Kif5B-driven peroxisome transport to the tip of neuronal leading process, where microtubule-plus-ends are supposed to be (**Figure 2-13. A, B**). In contrast, BicD2 confined peroxisomes to the MTOC, which was validated by immunostaining of γ -tubulin (**Figure 2-13. A, B**). Nesprin-2 SR48 Δ KASH generated active peroxisome movements bidirectionally, and immunostaining showed that peroxisomes were distributed around but not aggregating at microtubule minus ends (**Figure 2-13. A, B**). Consistent with observation in cell lines, Kif5B- or BicD2-driven peroxisome transport was quickly activated but decreased drastically within 20 min of rapalog addition, while SR48 Δ KASH kept the cargoes at a relatively active state persistently (**Figure 2-13. C**).

I next performed dual-color imaging with microtubules labelled by a DCX (doublecortin)-GFP construct, which is a microtubule-associated protein highly expressed in migrating neurons. Single peroxisomes moving on microtubule tracks could be traced (**Figure 2-13. D**). Kif5B- or BicD2-coupled peroxisomes moved rapidly toward the tip of leading process or toward the MTOC, respectively. SR48 Δ KASH generated bidirectional movements around the cell soma and in the proximal region of the leading process. Directional reversal of SR48 Δ KASH-driven cargo transport often occurred without a strong bias toward microtubule minus or plus ends (**Figure 2-13. D**).

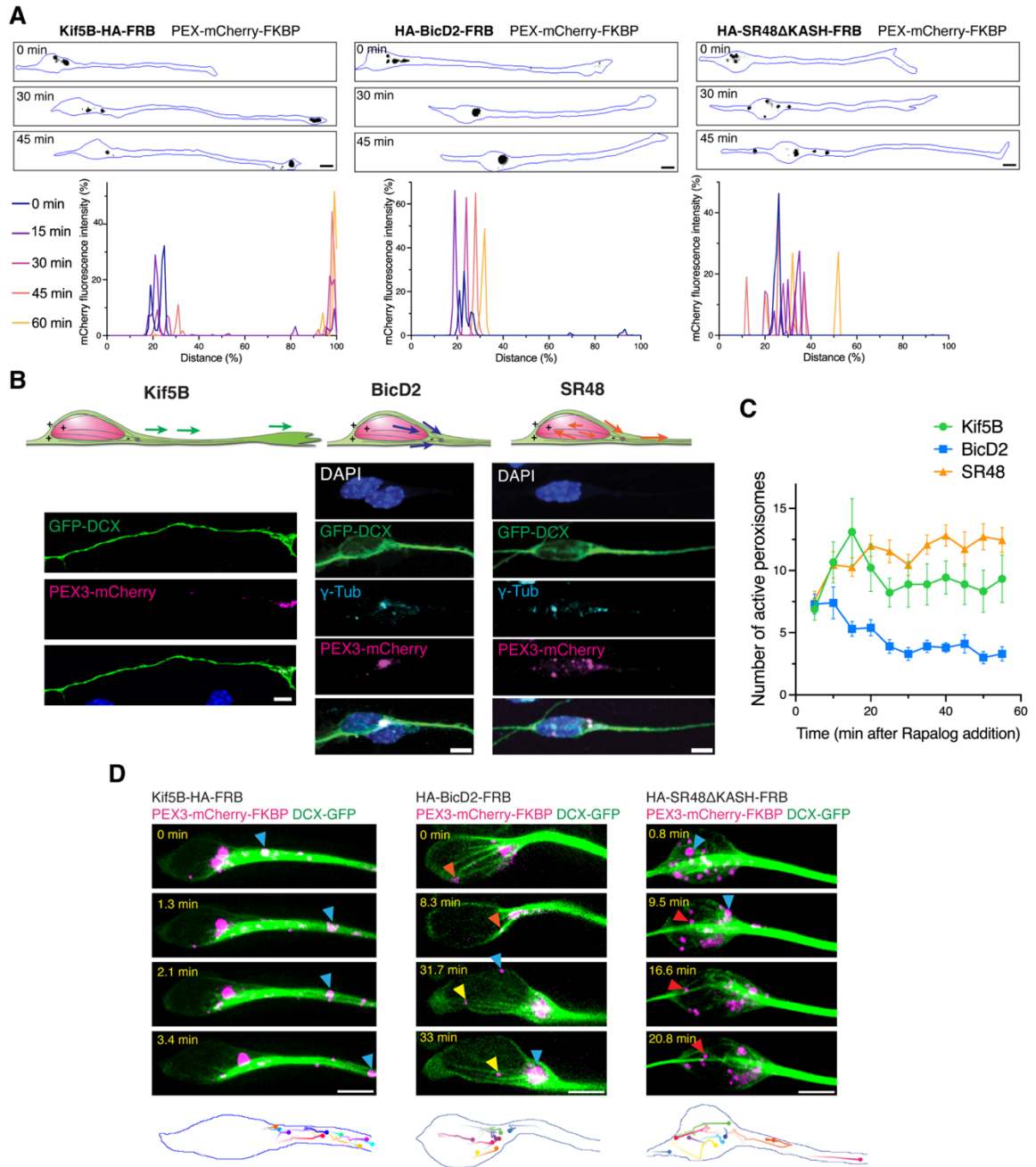


Figure 2-13. Nesprin-2 drives persistent bidirectional cargo transport in CGCs. (A) Inducible peroxisome trafficking assay in cultured CGCs transfected with PEX-mCherry-FKBP together with KIF5B-HA-FRB (*left*), HA-BicD2-FRB (*middle*), or HA-SR48ΔKASH-FRB (*right*). Images at 0, 30 and 60 min after rapalog treatment are shown. mCherry signals are shown in black and cell contours are outlined in blue. Lower graphs show the distribution of mCherry-labelled peroxisomes along the distance from the trailing process to the leading process at 0, 15, 30, 45 and 60 min after rapalog treatment. (B) *Top*, Schematic of peroxisome movement driven by Kif5B, BicD2 or Nesp2-SR48. *Bottom*, fixed samples showing peroxisome distribution at 1 hour after rapalog treatment. Kif5B transports peroxisomes toward the tip of the leading process. Immunocytochemistry staining with γ -tubulin (cyan) shows that BicD2 gathers peroxisomes at the centrosome, while SR48 distributes peroxisomes throughout the cell soma. (C) Quantification of the number of peroxisomes that

moved for 3 μm or more in respective time windows. $n = 9$ (Kif5B), 11 (BicD2) and 11 (SR48) cells from 3 independent experiments per group. Data points and error bars show mean $\pm$ SEM. **(D)** Time-lapse sequences showing peroxisomes (*magenta*) moving along DCX-GFP-labelled MT filaments (*green*) in CGCs. Peroxisomes transported by KIF5B-HA-FRB (*left*), HA-BicD2-FRB (*middle*), or HA-SR48 Δ KASH-FRB (*right*) are shown. Colored arrowheads trace the movement of individual peroxisomes. Bottom, representative trajectories of individual peroxisomes. Scale bars, 5 μm in (A, B and D).

2.3 Discussion

2.3.1 Nesprin-2 is required for nuclear translocation in migrating neurons

In Nesprin-2 mutant mice, cerebellar layer formation is disrupted, which is proposed to be caused by delayed migration of post-mitotic CGCs. It is in line with earlier studies showing that knocking out Nesprin-2 causes mis-localization of nuclei in retina and cerebral cortex (Zhang et. al., 2009; Yu et. al., 2011). Mutations of Nesprin-2 have also been implicated in human intellectual disability and autism diseases, which might be related to retarded neuronal migration as well (Young et. al., 2021). In my mutant mouse model, the genomic region targeted by CRISPR/Cas9-based editing is shared among all the nuclear-membrane-localizing isoforms of Nesprin-2, but such deletion does not abolish nuclear movements entirely. It might be due to partial complementary functions by other Nesprin family proteins like Nesprin-1 (which interacts with dynein), Nesprin-4 (which interacts with kinesin-1 and shows a high transcript level in cerebellum) or other nuclear envelope proteins like RanBP2 (Ran-binding protein 2) which interacts with cytoskeleton (Roux et. al., 2009; Taiber et. al., 2022; Yi et al., 2023). In addition to microtubule-based transport, the N-terminal region of Nesprin-2 may also assist mechanical transduction between nucleus and actin filaments (Zhen et. al., 2002). Future studies are needed to clarify different roles of these nuclear adaptor molecules, as well as interplay between microtubule-based and actomyosin-based transport mechanisms. It is very likely that different nuclear adaptors are active in different types of tissue during different developmental stages to establish correct nuclear positioning (Rajgor et. al., 2012). Nevertheless, in the case of CGC migration, SR48-KASH is sufficient to rescue nuclear migration defects caused by genetic ablation of Nesprin-2. By comparing wildtype and mutant constructs of SR48-KASH in rescue experiments, I demonstrated that recruitment of both dynein and kinesin-1 onto the nuclear envelope via Nesprin-2 resumed

normal nuclear movements while the single-motor-bound mutants behaved similarly as loss-of-function mutant.

2.3.2 Nesprin-2 interacts with dynein-dynactin-BicD2 complex and kinesin-1

Sequence alignment and site-specific mutation clarified close but distinct motifs for kinesin-1 and DDB binding in the membrane proximal region of Nesprin-2. The Spindly and CC1-box motifs are found in many dynein activators, with a CC1-box near the N-terminus, an extended coiled-coil domain in-between and a Spindly motif near the C-terminus, to mediate interactions with dynein light intermediate chain, Arp1 filament and pointed end complex of dynactin, respectively (Gama et al., 2017; Canty and Yildiz, 2020). In Nesprin-2, the positions of the putative motifs are reversed, and their arrangement is complex in the predicted protein conformation. My results also show that BicD2 is required for the interaction between Nesprin-2 and dynein-dynactin complex while the presumptive Spindly and CC1-box motifs on SR48-KASH are not sufficient for direct binding of dynein or dynactin, suggesting differential roles compared with the previously characterized Spindly and CC1-box motifs. Although the detailed mechanism remains elusive, the co-IP results of 5A-AAVV mutant protein indicate that the interaction between Nesprin-2 and DDB complex is dependent on these Spindly- and CC1-box-like sequence motifs, possibly by mediating BicD2 recruitment or maintaining certain protein conformation for DDB binding. The C-terminus of Nesprin-2 has been shown to bind to the C-terminal coiled-coil 3 (CC3) of BicD2, but the detailed interaction sites are still unknown (Tsai et al., 2020). Since BicD2 contains a kinesin-1-binding site, with much weaker binding affinities than BicD2-dynein-dynactin binding though, it was proposed that BicD2 mediates the concomitant recruitment of dynein-dynactin and kinesin-1 onto Nesprin-2 (Grigoriev et al., 2007; Gonçalves et al., 2020). As opposed to that hypothesis, my results show that kinesin-1 binding of SR48-KASH was unaffected by depletion of BicD2, suggesting that Nesprin-2 associates with kinesin-1 directly probably by binding to kinesin light chain (KLC) via the LEWD motif (Wilson and Holzbaur, 2015; Wu et al., 2018).

Notably, the predicted interface for interaction with DDB complex encompasses the kinesin-binding motif on Nesprin-2, similarly to other bidirectional motor adaptors including JIP1 and TRAK2 (Fu and Holzbaur, 2013; Fu et al., 2014; Van Spronsen et al., 2013; Fenton et al., 2021; Canty et al., 2023). Those bidirectional motor adaptors scaffold the complex involving

dynein-dynactin and kinesin, while differentially regulating the interplay of motors to achieve different distribution patterns of cargoes. JIP1 activates one motor for unidirectional transport toward either microtubule end depending on its phosphorylation state, resulting in rapid and long-range transport of amyloid precursor protein (APP) in neurons. In contrast, TRAK2 readily switches between opposing motors on a single microtubule, distributing intracellular mitochondria with high mobility and flexibility. While Nesprin-2 SR48ΔKASH is sufficient to induce both plus-end and minus-end-directed cargo transport, short episodes of transport to either direction is more prevalent than directional switching in my intracellular cargo transport assay. In between those processive transports, peroxisomes pause on the microtubules or detach and jump to another microtubule. Whether the switching depends on stochastic binding or more sophisticated regulation is unknown. Unidentified accessory proteins or kinase might play a role in determining directional switch. In fact, Klarsicht, the *Drosophila* KASH protein, was reported to coordinate bidirectional transport of lipid-droplet by forming a complex with Halo and LSD2 (lipid storage droplet protein 2) as molecular toggles (Welte et al., 1998; Welte et al., 2005).

2.3.3 Nesprin-2 supports sustained bidirectional cargo dynamics

I found that the velocity of minus-end-directed transport by Nesprin-2 is comparable to that of BicD2-driven transport, suggesting that Nesprin-2 may rely on the DDB complex for minus-end-directed cargo transport. On the other hand, Nesprin-2 enhances the velocity of plus-end-oriented transport compared to Kif5B, suggesting its activating function for kinesin-1. Although kinesin-1 is often thought to have rigid steps and consistent speed, emerging work suggests that its movements can be accelerated when associated with activating adaptors, including Nesprin-4, TRAK1 and JIP1 (Chiba et al., 2022; Henrichs et al., 2020; Chiba et al., 2017). My observation contrasts with the tug-of-war model which predicts reduced velocities for transport in both direction in the presence of opposing motors (Belyy et al., 2016), or the selective recruitment/activation model which predicts unaltered velocities of individual motors (Hendricks et al., 2010). My results suggest that Nesprin-2 coordinates interdependent functions of bidirectional motors. Such coordination mechanism has been demonstrated for TRAK2 which changes the microtubule landing rates of cargoes in the presence of opposing motors (Fenton et al., 2021; Canty et al., 2023). Several *in silico* studies also support that the involvement of multiple different motors affects association of cargoes

with microtubules, leading to changes of transport frequency and/or run length (Müller et al., 2010; Jensen et al., 2021). In contrast, Nesprin-2 bound with bidirectional motors showed equivalent run lengths, but enhanced speed compared to the mutants bound to only a single motor. It suggests that, rather than changing the association with microtubules, Nesprin-2 may mediate functional activation or disinhibition of motors by binding with the opposing motors. The Nesprin-2-motor complex lacking either motor might be in an unfavorable conformation to release the remaining motor from autoinhibition, and thus walk at suboptimal speed. In fact, motor velocities can be changed by adaptor molecules and intra-/inter-molecular tension between closely packed motors (Yildiz et al., 2008; Elshenawy et al., 2019). Besides, mechanical activation by opposing pulling force has also been proposed as an alternative co-dependent mechanism, awaiting further evidence (Ally et al., 2009; Hancock, 2014). In the intracellular cargo trafficking assay, it is hard to quantify the absolute frequency of active cargo transport and cargo interaction with microtubules due to the variation of local peroxisome abundance and microtubule density inside the cell. Future study is needed to determine whether the binding of opposing motor as structural scaffold or force generation by opposing motor as mechanical activation cue is required for motility activation.

I demonstrated in both cell lines and migrating CGCs that Nesprin-2 generated prolonged bidirectional transport compared to Kif5B- or BicD2-driven peroxisome transport. The latter quickly terminated at the cell periphery or cell center in a short time, resembling an “exhausted” state. In fact, *in vitro* studies have demonstrated that activated DDB or Kif5 motors do not dissociate quickly but strongly accumulate at microtubule minus or plus ends (Tan et al., 2018; Chiba et al., 2022). The persistence of movements generated by alternating dynein- and kinesin-1-driven transport might be beneficial for the large nucleus to navigate through crowded intracellular environment. Nesprin-2 may facilitate track switching at microtubule intersections or directional reversal when it encounters intracellular obstacles, as discussed in Chapter 1 (**Figure 1-5**). It is assumed that nuclear rotation, which is decreased in Nesprin-2-deficient neurons, is also an important aspect of nuclear dynamics for smooth transport (Wu et al., 2018).

2.3.4 Limitations and future directions

The nucleus is a large cargo and should simultaneously associate with a large number of motors. It has been reported that motor stepping and load-bearing capacity change when

larger cargoes are carried, or stronger resistant forces are applied (Rai et al., 2013; Pyrpassopoulos et al., 2020). In my cargo transport assay, directional preference of Nesprin-2-mediated transport was not affected by increasing the cargo size, negating that Nesprin-2 mediates generation of collective force of bound motors. However, it is challenging to determine the exact number of bound motors, active motors or the ratio of opposing motors in my assay. Stalling forces exerted by bidirectional motors via Nesprin-2 are also unknown. Future investigation by single-molecule imaging, biophysical experiments like optical trap, computational modeling and structural analysis of the Nesprin-2-motor macromolecule complex will be helpful to provide further understanding on how Nesprin-2 modulates motilities, force generation or attachment/detachment rates of dynein and kinesin-1.

While cell-free *in vitro* studies have widely been used in delineating the mechanism of bidirectional transport by DDB and kinesin motors, motility of intracellular native organelle is much more complex due to biophysical properties of membranous cargoes, intracellular co-factors or kinases, or local MT organization. Peroxisome kinetics in cell lines was analyzed successfully at sub-second accuracy, but such analysis could not be performed in CGCs due to technical difficulties in imaging through the thick neuronal cell soma at a fast rate. Advances of microscopic techniques or super-resolution imaging with faster speed will allow more precise characterization of cargo dynamics.

3 Chapter 3 – Nucleus is linked to forward-moving microtubules via Nesprin-2

3.1 Introduction

From **Figure 2-13**, I observed that, while the Nesprin-2-SR48 generated bidirectional movements, the peroxisomes gradually advanced forward as cell soma moved forward. These observations prompted me to hypothesize that organelles are not simply transported along stationary microtubule tracks, but they could be anchored onto forward-moving microtubules and travel together with the tracks.

The vast majority of studies on *in vitro* or intracellular cargo transport assumed that cargo delivery is solely attributed to motors walking on fixed microtubule tracks. This view has been challenged by a few studies. Kulić et al. (2008) proposed that movements of microtubule tracks also contributes to cargo transport after observing intracellular peroxisome

movements together with microtubules at a high temporal resolution. Another study using neurons also demonstrated that kinesin-3 motor Kif1A sequesters vesicular cargo onto microtubules for efficient delivery along with microtubule tracks that are transported into dendritic spines (McVicker et al., 2016). Such mechanism may explain the co-migration of various organelles toward the moving direction of microtubules, which adds an extra directional bias component to the bidirectional cargo movements on fixed microtubules.

It has been reported that microtubules undergoing dynamic rearrangement during neuronal morphogenesis, including microtubule polymerization, catastrophe and translational movements of short microtubules (Letourneau and Ressler, 1984; Kapitein and Hoogenraad, 2015). In highly polarized neuronal cells, microtubules are directionally transported to support neurite extension, form rails for cargo delivery, and also initiate axon regeneration upon injury (Baas et al., 2006; Baas and Mozgova, 2012; Rao et al., 2017; Miryala et al., 2022). However, whether microtubule mobility plays a role in neuronal nuclear migration is unclear yet. KifC1, a member of kinesin-14 family, was recently reported to slide and cross-link microtubules to facilitate nuclear movements during neuronal migration (Muralidharan et al., 2022). Thus, I hypothesize that Nesprin-2 anchors nucleus onto forward-moving microtubules, leading to predominantly forward nuclear movements in migrating CGCs.

3.2 Results

3.2.1 Nuclear migration is correlated with microtubule forward movements during neuronal migration

To track the movements of microtubules, I utilized a photo-convertible mEos2 probe fused with DCX in reaggregate culture of CGCs. mEos2 is a green-to-red photoconvertible fluorescence protein with high photostability and conversion efficiency (McKinney et al., 2009). Transfection with mEos2-DCX in CGCs labelled thick microtubule bundles in the leading process, separated from the thinner bundles in the trailing process (**Figure 3-1. A**). Upon laser illumination at a small region of the proximal leading process, a small subset of microtubules were demarcated by photoconversion of mEos2-DCX, the movements of which could be traced along with the nucleus labeled with SiR-DNA dye (**Figure 3-1. B**). I found that forward movements of the nucleus were often coupled with forward sliding of the labelled microtubule segments (**Figure 3-1. C**). It suggests that the nucleus is not transported forward along stationary microtubule tracks, but associated with forward moving microtubules.

3.2.2 Inhibition of Nesprin-2 disconnects nucleus and forward-moving microtubules

I next asked whether Nesprin-2 is involved in coupling the movements of nucleus and microtubules. Using the photo-conversion mEos2-DCX probe, I found that, in CGCs from Nesprin-2 mutant mice, nuclear movements were hampered, but microtubules still moved forward, leaving the nucleus behind (**Figure 3-2. A**). More severe disconnection between nucleus and microtubules was observed in CGCs over-expressing the dominant negative KASH construct (**Figure 3-2. B**), which was reported to block both Nesprin-1 and Nesprin-2 (Stewart-Hutchinson et al., 2008). Photo-converted MT segments moved forward at comparable speed among all groups, but the nucleo-microtubule (NM) distance gradually increased in Nesprin-2 mutant cells and KASH-over-expressing cells (**Figure 3-2. C, D**). Together, my data suggests that rather than simply dragging the nucleus towards microtubule minus-ends on fixed tracks, Nesprin-2 keeps the nucleus in a bidirectionally movable state while coupling it with forward-moving microtubules.

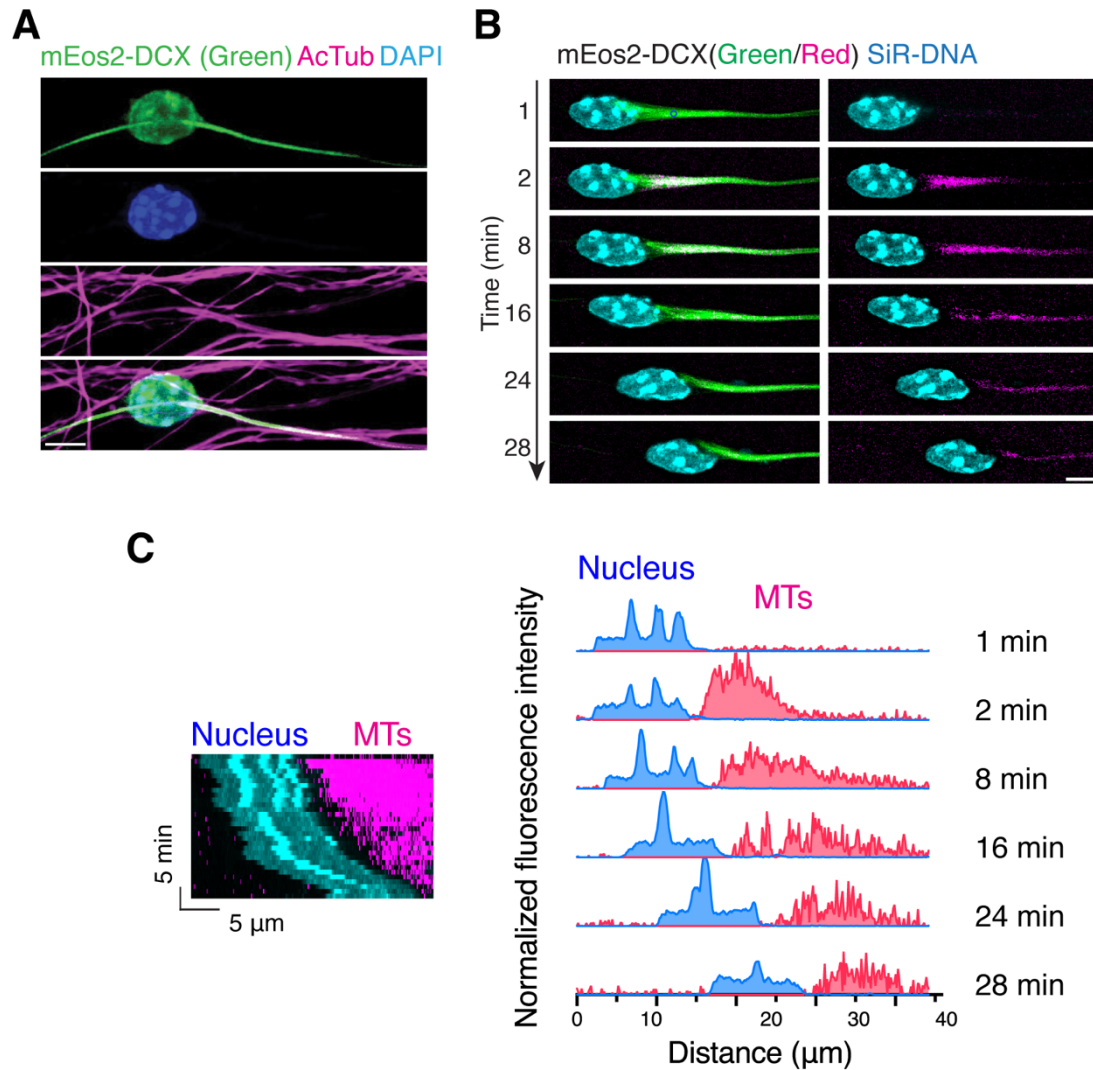


Figure 3-1. Forward nuclear translocation is accompanied with forward movements of microtubule tracks. (A) Immunocytochemistry staining of CGCs transfected with mEos-DCX. mEos-DCX (*green*) is colocalized with acetylated-tubulin (*magenta*). (B) Time-lapse sequences of a representative CGC in reaggregate culture. Cells were transfected with mEos2-DCX (*green* or *red*). The nucleus was labelled with SiR-DNA (*cyan*). At 2 min, a strong 405-nm laser was applied at a point (denoted by a blue circle) in the proximal region of the leading process and mEos2-DCX underwent green-to-red photoconversion. (C) Kymograph (*left*) and intensity distribution profile (*right*) of fluorescent signals of the cell from (B). Synchronous forward movement of the nucleus (*blue*) and the photo-converted MTs (*red*) was observed. Scale bars, 5 μ m in (A, B).

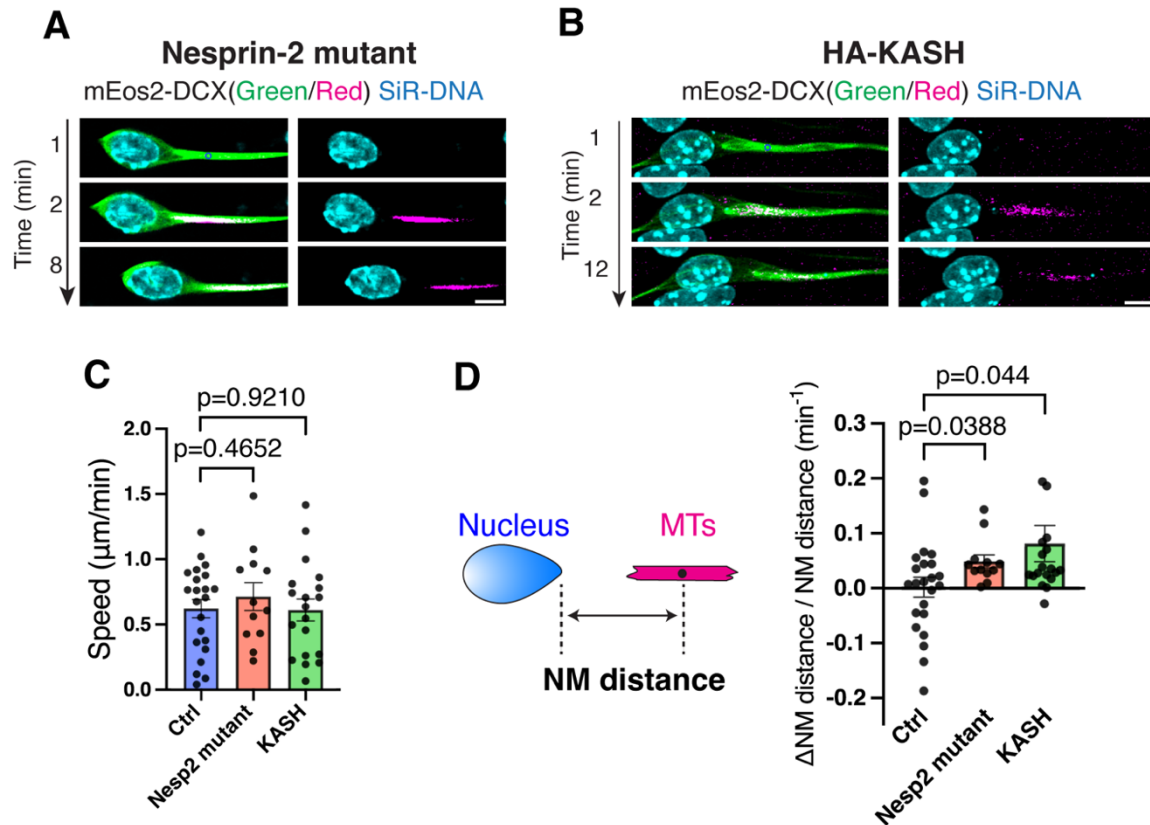


Figure 3-2. Nesprin-2 dysfunction causes disconnection between nucleus and forward-moving microtubules. (A) Time-lapse sequences of a representative CGC from Nesprin-2 mutant mice. (B) Time-lapse sequences of a representative WT CGC overexpressing HA-KASH. Photoconversion is induced at 2 min at the points demoted by blue circles. Cells were transfected with mEos2-DCX (green or red) and the nucleus was labelled with SiR-DNA (cyan) in (A) and (B). At 2 min, a strong 405-nm laser was applied at a point (denoted by a blue circle) in the proximal region of the leading process and mEos2-DCX underwent green-to-red photoconversion. (C) Quantification of MT forward moving speed. Unpaired t test was used to compare to WT (Ctrl). (D) Quantification of changes in Nucleo-Microtubule (NM) distance in migrating CGCs. The change of NM distance over 3 ~ 6 min is normalized to the initial NM distance. Unpaired t test with Welch's correction. n = 22 (WT), 12 (mutant) and 19 (KASH) cells from 2 ~ 6 independent experiments per group. Bars show mean $\pm$ SEM. Scale bars, 5 μm in (A, B).

3.3 Discussion

I found that forward microtubule movements are utilized to direct forward nuclear transport via anchorage by Nesprin-2. Although nuclear-centrosome (N-C) coupling is widely used to explain that neuronal nucleus is linked to centrosome for forward directed translocation (Solecki et al., 2004; Bellion et al., 2005; Schaar and McConnel, 2005; Tsai et al., 2007), I propose here that nucleo-microtubule coupling also plays a critical role for nuclear transport in unison with forward-moving cytoskeleton. This coupling mechanism should be different from passive hitchhiking, where cargo can only attach or dettach from the moving tracks. In the scenario of Nesprin-2-mediated anchorage, nucleus exhibits dynamic movements like rotation and small bidirectional motion, until a substantial leap forward is achieved when a group of peri-nuclear microtubules advances forward. It provides a novel mechanism for how net direction of transport can be achieved with bidirectional adaptor. The previously characterized saltatory motion of neuronal nucleus might be associated with the timing of microtubule forward sliding (Umeshima et al., 2007). This might be a more general mechanism to facilitate the transport of other intracellular organelles as well in migrating cells.

Trivedi et al. (2017) also reported that microtubules are moving forward during neuronal migration, and the speed of microtubule movements from my results ($0.62 \pm 0.33 \mu\text{m}/\text{min}$ in wildtype CGCs) is similar to their study ($0.66 \mu\text{m}/\text{min}$). It has been reported that forward movements of microtubules in neurons requires actin-microtubule cross-linking (Hutchins and Wray, 2014; Trivedi et al., 2017), and dynein- or kinesin-driven microtubule sliding (Rao et al., 2016; Muralidharan et al., 2022; Schelski and Bradke, 2022). There is a considerable amount of perinuclear microtubules that are detached from centrosomes in migrating neurons (Rao et al., 2016; Wu et al., 2018), but, from my data, I couldn't judge whether microtubules slide forward collectively with centrosomes, or the non-centrosomal microtubules are undergoing forward sliding independently from centrosomes. Aside from sliding motors, kinesin-5, kinesin-6 and kinesin-12 can also act as brakes to stop microtubule movements during neuronal migration (Myers and Baas, 2007; Liu et al., 2010; Del Castillo et al., 2015). Further investigation may reveal further controlling mechanisms for microtubule mobility.

4 Chapter 4 – Concluding thoughts and future outlook

Taken together, Nesprin-2 incorporates the motilities of dynein and kinesin-1 motors to generate persistent bidirectional cargo movements. Unlike the prevailing view that dynein acts as a predominant motor propelling nuclear transport along stationary microtubules, I propose that the nucleus continues small-step bidirectional movements along the forward moving perinuclear microtubules (**Figure 4-1**). My study provides a novel perspective on the long unsolved paradox of motor co-dependence. In the future, characterization of Nesprin-2 transport at single molecular level or protein structure analysis of Nesprin-2-motor complex should reveal further mechanisms of regulation.

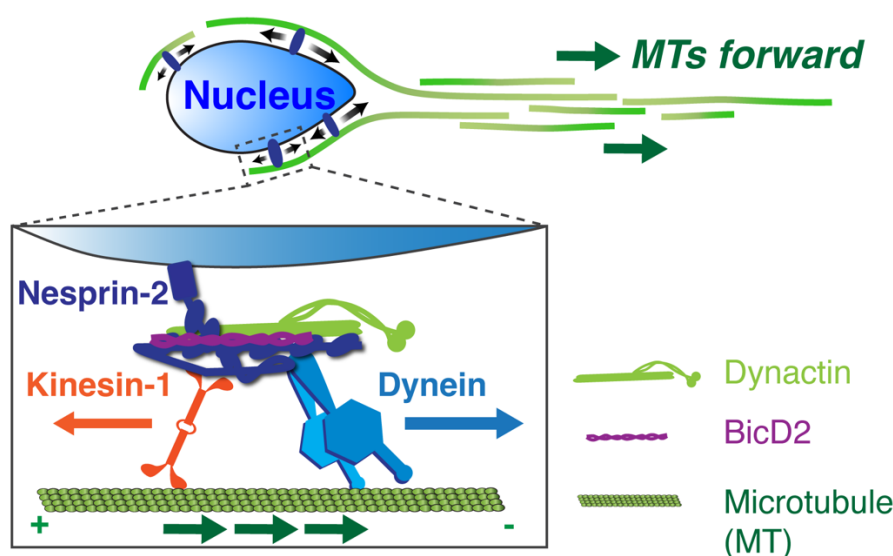


Figure 4-1. Proposed mechanism of Nesprin-2-driven neuronal nuclear translocation. Forward movements of the nucleus in CGCs are generated by the combination of persistent bidirectional transport mediated by Nesprin-2 together with MT forward movements.

In addition to neuronal migration, the positioning of nucleus is actively regulated in other basic cellular processes like cell division and differentiation. Nesprin-2-mediated nuclear dynamics occurs not only in developing brain, but also in diverse cell types, including

migratory immune cells, invasive cancer cells, cardiac and skeletal muscle cells (Gundersen and Worman, 2013). This study provides new insights into motor-nuclear interactions under both physiological and pathological conditions, potentially aiding in the fight against diseases related to abnormal nuclear motility and positioning. For instance, it may help identify precise molecular targets to combat cancer cell invasion with less side effects.

From a broader perspective, exploring the mechanisms of adaptor function and cargo transport can deepen our understanding of neurodevelopmental and neurodegenerative diseases. For example, Alzheimer's disease is marked by the formation of tau protein tangles, which disrupt axonal transport (Millecamps and Julien, 2013). Similarly, amyotrophic lateral sclerosis, Huntington's disease, and Parkinson's disease are thought to involve impairments in anterograde and retrograde axonal transport (Sleigh et al., 2019). Elucidating the functional regulation between adaptor molecules and motors will provide novel approaches for rehabilitating cargo transport in affected neurons.

5 Materials and Methods

5.1 Animals

All mice were treated in accordance with the guidelines of the Animal Experiment Committee of Kyoto University and the 3R principles (Replacement, reduction and refinement) was followed. Wild-type ICR mice (Slc:ICR) were supplied by Japan SLC. During experiments, anesthesia was performed by ice-induced cryoanesthesia for neonatal mice, or isoflurane inhalation for adult mice to minimize pain. Mice were used without distinction between male and female.

5.2 Plasmids and reagents

Plasmid constructs used in this thesis are listed in Table 1. In-Fusion cloning (Takara) with self-designed primers (Eurofins JP) was used for ligating large DNA fragments (>50 bp) while T4 ligase (TOYOBO) was used for ligating small DNA fragments (< 50 bp).

For Nesprin-2 expression plasmids, the SRs of mouse Nesprin-2 were defined by the UniProt database (UniProt Consortium, 2023) and the NCBI database. Nesprin-2 fragments were cloned from a mouse cDNA library and inserted into entry vectors to be fused with different protein tags on destination vectors by the Gateway recombination method. The pENTR1A vector (Invitrogen) was used and Nesprin-2 sequences were inserted between the attL1 and attL2 sites. Nesprin-2 SR1 ~ SR56 were cloned except for SR17~SR20 due to difficulty in cloning this segment from our available cDNA libraries. Destination vectors were constructed by inserting the Gateway cassette with the attR1 and attR2 sites into pCAGGS vector to make pCAG-Dest. Halo tag was cloned from pHTN HaloTag[®] CMV-neo (Promega) and fused with an HA tag before being inserted into the pCAG-Dest vector (pCAG-Halo-HA-Dest). mNG sequence was amplified from pmNeonGreen-C1 and inserted into the pCAG-Dest vector (pCAG-mNG-Dest). Gateway LR recombination was performed between pENTR1A-Nespr2-SRs plasmids and pCAG-Halo-HA-Dest or pCAG-mNG-Dest plasmids using Gateway LR clonase (Invitrogen) to generate plasmids expressing Halo-HA- or mNG-fused Nesprin-2 fragments as listed in Table 1. Site-specific mutations of SR48-5A-AAVV or SR48-LEAA were introduced using primers containing the desired mutations by In-Fusion Cloning (Takara).

For peroxisome trafficking assay, the FKBP- and FRB-containing plasmids (Table 1) were constructed by modifying the original FKBP and FRB plasmids gifted by Professor Lucas Kapitein. Kif5B (1-807aa), BicD2 (1-595aa) and tubulin alpha 1b (α -tubulin) were cloned from a mouse cDNA library. SR48 Δ KASH (5655-6767aa) was cloned from the pENTR1A-SR48KASH plasmid. pGW2-FRB-HA-DHC was made by replacing the GFP on pGW2-FRB-GFP-DHC (gift from Kapitein) to HA tag. FRB and FKBP constructs were expressed by pGW which contain a CMV promoter in COS7 and MRC5 cell, while equivalent constructs were expressed by pCAG which contain a CAG promoter for a higher expression level in neurons. mVenus-SKL was constructed by fusing mVenus tag with a short sequence expressing Ser-Lys-Leu (5'-TCCAAACTC-3').

For photo-conversion experiment, DCX was cloned from pCAG-DCX-GFP and fused with mEos2.

Table 1. Plasmids

Plasmid	Source
pCAG-EGFP	Wu et. al., 2018
pCAG-DsRed-NLS	Wu et. al., 2018
pCAG-GFP-Lis1N	Wu et. al., 2018
pCAG-GFP-Kif5B-tail	Wu et. al., 2018
pCAG-Halo-HA-Nesp2-SR1~11/ 12~16/ 21~31/ 32~40/ 41~50/ 48~56	This paper
pCAG-Halo-HA-Nesp2-SR55/ 53/ 52/ 49/ 48-KASH	This paper
pCAG-Halo-HA-Nesp2-SR48-5A-AAVV-KASH	This paper
pCAG-Halo-HA-Nesp2-SR48-LEAA-KASH	This paper
BICD2 CRISPR/Cas9 KO plasmid	Santa Cruz (sc-405525)
pCAG-mNG-SR48-KASH	This paper
pCAG-mNG-SR48-5A-AAVV-KASH	This paper
pCAG-mNG-SR48-LEAA-KASH	This paper

pCAG-GFP-EB3	Wu et. al., 2018
pGW1-PEX3(1-42aa)-GFP-FKBP	This paper
pGW1-Kif5B(1-807aa)-HA-FRB	This paper
pGW1-HA-BICD2(1-595aa)-FRB	This paper
pGW1-SR48ΔKASH-FRB	This paper
pGW1-SR48-5A-AAVV-ΔKASH-FRB	This paper
pGW1-SR48-LEAA-ΔKASH-FRB	This paper
pAAV-CAG-mCherry-α-Tubullin	This paper
pGW2-FRB-HA-DHC	This paper
pGW1-mVenus-SKL	This paper
pAAV-CAG-PEX-mCherry-GM130 ^{C-ter}	Fukumitsu et. al., 2015
pCAG-PEX3-mCherry-FKBP	This paper
pCAG-Kif5B(1-807aa)-HA-FRB	This paper
pCAG-HA-BICD2(1-595aa)-FRB	This paper
pCAG-HA-SR48ΔKASH-FRB	This paper
pCAG-DCX-GFP	Wu et. al., 2018
pCAG-mEos2-DCX	This paper
pCAG-HA-KASH	Umeshima et. al., 2007

5.3 Organotypic and primary culture of CGCs

In vivo electroporation and lobe culture were performed as described previously with a few modifications (Umeshima et al., 2007; Umeshima and Kengaku, 2013). Briefly, DNA solution (0.7 ~ 1 μ l of 6 μ g/ μ l per mouse) was injected into the cerebellum of P5 mice and electric pulses were applied by a pulse generator (CUY21 Nepagene) through a hook-type electrode attached on the injection needle and a tweezer-type electrode on the head. At P6, mouse brains were dissected, and the cerebellum lobes labelled with fluorescence protein expression were isolated gently in artificial cerebrospinal fluid (aCSF) bubbled with a gas mixture of 95% O₂ and 5% CO₂. The dissected cerebellar lobe IV, V or VI were then plated

on Millicell cell culture insert (Millipore) and covered by a thin layer of collagen gel. Before and during imaging, the culture was maintained in culture medium (5.5 g/L Basal Medium Eagle (BME) (Sigma-Aldrich), 25% Earle's Balanced Salt solution (Sigma-Aldrich), 15% heat-inactivated horse serum (Thermo Fisher Scientific), 5.6 g/L glucose, 3 mM GlutaMAX™ Supplement (Gibco), 1.8 g/L sodium bicarbonate, 1 mM sodium pyruvate, 1% N2 supplement (Gibco) and 50 units/mL Penicillin-Streptomycin (P-S)). Time-lapse imaging was performed 3~18 hours after culture preparation.

Reaggregate culture was performed as described previously (Wu et al., 2018). Reaggregate culture was maintained in Mason's medium (9.2 g/L BME (Sigma-Aldrich), 2.1 g/L sodium bicarbonate, 4.76 g/L glucose, 10 g/L BSA, 1% N2 supplement (Gibco) and P-S). Time-lapse imaging to observe granule cell migration was performed 18~30 hours after plating of the reaggregates.

5.4 Time-lapse imaging of CGCs

Live-cell imaging was performed as described previously (Umeshima et al., 2007; Wu et al., 2018) with some modifications.

For lobe cultures, time-lapse imaging of 30-s intervals was performed on a laser-scanning confocal microscope (Olympus FV1000-BX61WI) with high-sensitivity GaAsP detectors. Samples were kept in a stage-top incubator with temperature at 37°C and gas flow of 85% O₂, 5% CO₂ and 10% air. A 20x (N.A. 0.5) and a 40x (N.A. 0.8) water-immersion objective were used. The FV10-ASW Viewer software (Olympus) was used for image acquisition.

For reaggregate culture, time-lapse imaging of 30-s intervals was performed on a spinning-disk confocal microscope system (Yokogawa CV1000) with an enclosed incubator box. Samples were kept at 37°C with 5% CO₂ flow. Observation was through a 100x (N.A. 1.3) oil-immersion objective lens. Images were acquired through the CellVoyager CV1000 software (Yokogawa).

5.5 Isoform analysis and protein structure prediction

Nesprin-2 isoform analysis was performed by the MISO model based on Katz et al. (2010) and Trapnell et al. (2012). The bulk RNA sequencing data of CGCs was unpublished data

from a lab colleague Zhejing Zhang. The 3D structures of Nesprin-2 proteins were generated by ColabFold (Mirdita et al., 2022). The coiled-coil prediction was generated by Deepcoil (Ludwiczak et al., 2019).

5.6 Cell line culture

HEK293T cells (Riken BRC Cell Bank) were maintained in DMEM (Thermo Fisher Scientific) with 10% heat-inactivated FBS and P-S. COS7 and MRC5 cells (RIKEN BRC Cell Bank) were maintain in a 1:1 mixture of DMEM: Ham's F-10 Nutrient Mix (Thermo Fisher Scientific) with 10% heat-inactivated FBS and P-S. All cells were kept in 37°C incubator with 5% CO₂. Cells were passaged every 3 ~ 4 days before 100% confluency and kept on surface-treated culture dishes (Nunc dishes by Thermo Fisher Scientific or TC-treated dishes by Corning).

5.7 Co-immunoprecipitation and Western Blot

Co-immunoprecipitation was performed based on the product datasheet of HaloLink Resin (Promega) with a few modifications. HEK293T cells were transfected with Lipofectamine 2000 for 40~48 hours to express Halo-tagged Nesprin-2 protein before harvest. Cells were lysed on ice with PHEM buffer (50 mM PIPES, 50 mM HEPES, 1 mM EGTA, 2 mM MgSO₄) supplemented with Halt[®] protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and 1% Digitonin (FUJIFILM Wako Pure Chemical) as a gentle detergent. Cell lysate was cleared by centrifuging at 17000 g at 4°C for 15 min. Supernatant was saved as input lysate and protein concentration was measured by protein assay BCA kit. Equal amount of lysate with 1 µg/µl of total protein was prepared as input samples and each sample was incubated with 50 µL of pre-washed HaloLink Resin at 4°C for 2 ~ 4 hours. Beads were washed with PHEM buffer with 1 mg/mL BSA for 5 times (5 min each wash) to reduce non-specific binding. SDS sample buffer was used for elution and the elute was analyzed as IP samples by SDS-PAGE and Western blot.

For Western blot, Primary antibodies used are listed in Table 2. Membranes were cut according to protein ladder and membranes were stained with anti-DHC, p150, KHC and BicD2 antibodies separately. Stripping was performed with WB Stripping Solution (Nacalai tesque) by incubation at 60°C for 20 ~ 25 min, and re-probing was performed with anti-Halo

antibodies to check pull-down efficiency. HRP-conjugated secondary antibodies (Bio-Rad Laboratories) were used, followed by chemiluminescence reaction with Amersham™ ECL™ Select kit (Cytiva). Images of blots were taken by a gel imager (FUJIFILM LAS-4000) through the ImageQuant LAS 4000 Control Software. Results were analyzed by the gel analyze function on FIJI.

Table 2. Antibodies and reagents

Antibody or reagent	Source	Application and dilution
Lipofectamine 2000	Invitrogen	Transfection
Anti-Halo	G9281 (Promega)	WB 1:1000
Anti-DYNC1H1 (DHC)	12345-1-AP (Proteintech)	WB 1:500
Anti-p150[Glued]	610473 (BD Biosciences)	WB 1:1000
Anti-UKHC F-5 (KHC)	Sc-133184 (Santa Cruz)	WB 1:1000
Anti-BICD2	Ab117818 (Abcam)	WB 1:1000
Anti-Nesprin2	PA5-78438 (Invitrogen)	WB 1:1000; IHC and ICC 1:200
HRP-conjugated anti- β -actin	Sc-47778 (Santa Cruz)	WB 1:1000
Anti-p27-Kip1	610241 (BD Biosciences)	IHC 1:400
Anti-Ki-67	14-5698-82 (eBioscience)	IHC 1:1000
Anti-laminB1	Ab16048 (Abcam)	ICC 1:1000
Anti- γ -tubulin	T5192 (Sigma-Aldrich)	ICC 1:250
Anti- β -tubulin	T4026 (Sigma-Aldrich)	ICC 1:1000
Anti-acetylated-tubulin (6-11B-1)	T6793 (Sigma-Aldrich)	ICC 1:1000
SiR-DNA	CY-SC007 (Cytoskeleton)	Live-cell imaging 200 nM

5.8 Establishment of BicD2 knockout cell lines

Knockout cell lines were generated as described by Ran et al. (2013) and Han et al. (2022). HEK293T cells were transfected with BICD2 CRISPR/Cas9 KO plasmids by Lipofectamine for 2 days prior sorting. Cells were dissociated into single cells and FACS sorting was performed to collect the GFP-positive cells. The sorted cells were re-plated onto culture dishes sparsely and the culture was expanded for 10 days. Cell colonies could be identified by visual inspection. In total, 10 colonies were isolated through trypsin digestion and re-plated into sperate culture dishes for further expansion. SDS-PAGE and Western Blot was performed using lysate from the isolated cell clones to examine knockout efficiency, among which 5 out of 10 clones showed depletion of BicD2 protein (data not shown). The two cell clones with faster growth rate were used in co-immunoprecipitation experiments.

5.9 Establishment of Nesprin-2 mutant mouse line

Nesprin-2 mutant mouse line was generated by i-GONAD method using ICR mice according to Ohtsuka et al. (2018) and Gurumurthy et al. (2019). CRISPR RNA targeting the 108th exon of Syne2/Nesprin-2 (crRNA sequence 5'-GTAAAGCTGCTATTACGTCAAGG-3') and trans-activating crRNA (tracrRNA sequence 5'- CCTTGACGTAATAGCAGCTTTAC-3') were designed by a web tool CHOPCHOP (Labun et al., 2019). RNP complexes of gRNA duplexes and Alt-R™ S.p. Cas9 Nuclease V3 (Integrated DNA Technologies) were injected into the oviduct of 0.7-day pregnant mice and electric pulses were applied by electroporator (NEPA21) to introduce the RNP complexes into zygotes at the two-cell stage. The electroporated mother mice then gave birth to G0 founders. Genotyping was performed for all mice at P4 ~ P9 based on the method reported by Bhattacharya and Van Meir (2019). Genome DNA was extracted from mouse tail or finger samples with DirectPCR® DNA Extraction System (Viagen). Genotyping was performed with forward (5'- GTTGTGTTGGGAATTGTTTCACAGA-3') and reverse (5'- CAGACACCAAGCTCCACATATC-3') primers. The G0 mice carrying insertion or deletion were mated with wild-type mice to obtain G1 offspring. The PCR products from G1 mouse genotyping preparation were sequenced. A mouse line carrying a 16-bp deletion was selected. Such deletion was expected to generate frame shift and a novel pre-mature stop codon 30-bp downstream from the start position of the 108th exon. Hence, the mouse line either expresses

a truncated form of Nesprin-2 without the C-terminal KASH domain or shows an overall reduction of Nesprin-2 translation by nonsense-mediated decay of Nesprin-2 mRNA.

To verify the depleted Nesprin-2 expression in our mutant mouse line, lysate of cerebellum tissue from P9 pups was prepared in RIPA buffer and Western blot was performed using NuPAGE™ 3 to 8% Tris-Acetate protein gel (Thermo Fisher Scientific) for better visualization of proteins with large molecular weights.

After validation of mutant mouse line, heterozygous mice were inter-crossed to obtain homozygous wild-type and homozygous mutant littermates for histology phenotyping and primary culture.

5.10 Immunohistochemistry and analysis of cerebellum morphology

For cerebellum sample preparation, anesthetized mice were perfused with 4% PFA in phosphate buffer (PB). Dissected brains were postfixed for 3 hours with 4% PFA in PB followed by washing in PBS. The fixed brains were dehydrated in 15% sucrose in PBS for 6~10 hours, and then in 30% sucrose for overnight at 4°C. Brains were embedded in O.C.T. compound for snap freezing. Midsagittal sections of 10-μm thickness were prepared with Cryostat (Leica CM1950). For Immunohistochemistry, brain sections were treated with HistoVT One (Nacalai tesque) at 70°C for 20min for antigen retrieval. After permeabilization and blocking, slices were incubated with primary antibodies overnight at 4°C, followed by incubation with Alexa Fluor secondary antibodies at 4°C for 8~10 hours. Primary antibodies used are listed in Table 2. Images were acquired with a laser-scanning confocal microscope (Olympus FV1000-BX61) through 40x (N.A. 0.95) or 100x (N.A. 1.4) oil immersion objective lenses. For Nissl staining, cryosection slices were stained in 0.1% Cresyl Violet solution at 37°C for 5 min. After dehydration and clearing, slices were mounted with Entellan™ new (Sigma-Aldrich). Images were taken by an upright optical microscope (Leica DM5000B) with 2.5x (N.A. 0.07), 10x (N.A. 0.3) and 20x (N.A. 0.5) objective lenses through a AdvanCam-U3X camera (Advan Vision) and the AdvanView imaging software.

5.11 Immunocytochemistry of CGC primary cultures

For staining with Nesprin-2 or γ -tubulin, reaggregate culture samples were fixed with ice-cold methanol at -20°C for 5 ~ 10 min. Primary antibody incubation was performed at 4°C overnight. After washing, samples were incubated with Alexa Fluor secondary antibodies at room temperature for 1 hour, followed by DAPI staining ($10\text{ }\mu\text{g/mL}$) and mounting with ProLong™ Gold Antifade Mountant (Invitrogen).

For staining with acetylated-tubulin, samples were fixed in 4% PFA with 0.1% glutaraldehyde in PHEM buffer at room temperature for 20 min, followed by permeabilization with 0.2% Triton X-100 in PHEM buffer for 15 min. The following blocking and antibody incubation were performed in PHEM buffer with the same incubation temperature, time, and mounting method as the methanol-fixed samples above. Primary antibodies used are listed in Table 2. Images were acquired by the same microscope system as described above for immunohistochemistry samples.

5.12 Drug-inducible peroxisome trafficking assay

Peroxisome trafficking assay was performed as described by Kapitein et al. (2010) with some modifications. COS7 cells were plated on glass-bottom dishes (IWAKI) coated with $50\text{ }\mu\text{g/mL}$ fibronectin (Sigma-Aldrich; coating at 37°C for 1 hour). MRC5 cells were plated on glass-bottom dishes (IWAKI) coated with 0.2 mg/mL poly-D-lysine (coating at 37°C for 1 hour). Cells were transfected with indicated plasmids and Lipofectamine 1 day after plating. Cargo trafficking assay and live-cell imaging was performed 1 day after transfection for COS7 cells and CGCs, or performed 2 days after transfection for MRC5 cells. To induce peroxisome movements, A/C Heterodimerizer (also called rapalog AP21967; Clontech Laboratorie) was added at a final concentration of 500 nM in COS7 cells, $1\text{ }\mu\text{M}$ in MRC5 cells and 100 nM in CGCs. For COS7 cells and CGCs, time-lapse imaging of 30-s or 5-s intervals was performed by a spinning-disk confocal microscope system (Yokogawa CV1000) with a 40x oil-immersion (N.A. 1.3) or a 100x oil-immersion (N.A. 1.3) objective lenses. For MRC5 cells, dual-color time-lapse imaging was performed using a multimodal fast confocal microscope system (Andor Dragonfly 500) with a 100x oil-immersion (N.A. 1.49) objective lens. Images of both channels on a single z-plane were acquired simultaneously by a EMCCD camera (iXon Ultra) and a sCMOS camera (Zyla 4.2 Plus) at

102-ms intervals with the “Finite Burst” mode and the imaging was performed within 30 min after rapalog addition. All cells were imaged at 37°C with 5% CO₂ gas flow in culture medium.

5.13 Photoconversion experiments in CGCs

Reaggregate cultures were incubated with 200 nM of SiR-DNA dye for at least 2 hours before imaging. Triple-color time-lapse images were acquired by a laser-scanning confocal microscope (Zeiss LSM880) with the ZEN Black software (Zeiss) at 1-min intervals through a 40x oil-immersion (N.A. 1.4) objective lens. Photoconversion was induced by applying strong 405nm laser (laser power 45%) at a spot in the proximal region of CGC leading process for 1 iteration (2 ~ 3 s). The magenta channel shown in the figure was the original red fluorescence channel subtracting the pixel values of the far-red fluorescence channel so that the noise from the SiR-DNA signal was minimized.

5.14 Image analysis and data presentation

All Images were processed and analyzed using the FIJI software (Schindelin et al., 2012). For fluorescence images, maximum projections of multiple z stacks were used in all figures except for the following data: (1) dual-color imaging of single peroxisomes on microtubules was acquired on a single z-plane (**Figure 2-10. A-E; Figure 2-11. C, D**); (2) fixed samples stained with Lamin B1 and Nesprin-2 was shown as images of single z-planes to visualize the nuclear rim (**Figure 2-6. E**). Gamma adjustment with value 0.5 was performed on images in **Figure 2-10. B-E** and **Figure 2-11. C, D** for better visualization of microtubule filaments. Prism 10 (GraphPad) and MATLAB R2023b were used for plotting trajectories as well as quantitative analysis.

For analysis of nuclear movements, the centroid position of nuclei at each time frame was detected by the “Analyze Particles” function on FIJI. Net displacement of the nuclei was calculated from the distance between initial and final nuclear positions. Total travel distance was the summation of both forward or backward nuclear movements between each time frame.

For peroxisome trafficking assay in COS7 and CGCs, peroxisome movements were tracked by a particle tracking tool from the MosaicSuite plug-in on FIJI (Sbalzarini and Koumoutsakos, 2005) with the following parameters: kernel radius 0.1 ~ 1 μm , cutoff score 0.01, intensity percentile 0.1 ~ 0.5, particle link range 1 frame, maximum step length 7 μm , and Hungarian optimizer. A custom MATLAB program was used to plot the representative trajectories with the cell outline and another custom MATLAB program was used to analyze peroxisome dynamics at different time points. The number of active peroxisomes at each time windows were counted as the number of trajectories that travel for more than 3 μm in a direction within 45° parallel to the radius of the cell. Peroxisomes at the cell edges were excluded due to random movements caused by the active polymerization and depolymerization of microtubule filaments at the cell periphery. To analyze the distribution of peroxisomes in the cell, the pixel intensities of PEX-GFP-FKBP or PEX-mCherry-FKBP signal were summed up along the distance from the cell center to the cell edge using a custom MATLAB program. The center and outline of the cell were determined from the co-expression of mCherry- α -tubulin and the center was identified as the point where microtubules converged in the peri-nuclear region. Directions of EB3 comets were analyzed using KymoButler (Jakob et al., 2019).

To analyze the velocities and run lengths of peroxisomes from fast acquisition images in MRC5 cells, peroxisomes that moved for over 1 μm along a single microtubule were manually identified and kymographs were generated by tracing the microtubule filaments with orientation from the cell center to the cell edge through a semi-automated FIJI plug-in KymoAnalyzer (Neumann et al., 2017). Quantification of velocities and run lengths was also performed using KymoAnalyzer by selecting the kymograph segments with a constant slope, which were regarded as movements with constant speed. The representative kymographs shown in **Figure 2-10. B-E** and **Figure 2-11. C-D** were traced with a FIJI toolbox SNT (Arshadi et al., 2021). The values of run lengths were fitted by an exponential decay curve on MATLAB.

To analyze the microtubule movements in CGCs, a 3 ~ 6-min image where the mEos2(Red)-labelled microtubules moved forward was selected from each cell. The speed of microtubule movements was calculated from the initial and final centroid positions of the photo-converted microtubule segments. The NM distance was calculated as the distance between the front of the nucleus and the centroid of the photo-converted microtubules.

5.15 Statistical tests

All statistical analysis was performed using Prism 10 (GraphPad). Figures and figure legends contain the statistical test used and specific P values for each quantification. F-test of equality of variances was performed to determine whether unpaired t test or unpaired t test with Welch's correction was used.

6 Bibliography

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