Video Article

Validation of a Mouse Model to Disrupt LINC Complexes in a Cell-specific Manner

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Abstract

Nuclear migration and anchorage within developing and adult tissues relies heavily upon large macromolecular protein assemblies called LInkers of the Nucleoskeleton and Cytoskeleton (LINC complexes). These protein scaffolds span the nuclear envelope and connect the interior of the nucleus to components of the surrounding cytoplasmic cytoskeleton. LINC complexes consist of two evolutionary-conserved protein families, Sun proteins and Nesprins that harbor C-terminal molecular signature motifs called the SUN and KASH domains, respectively. Sun proteins are transmembrane proteins of the inner nuclear membrane whose N-terminal nucleoplasmic domain interacts with the nuclear lamina while their C-terminal SUN domains protrudes into the perinuclear space and interacts with the KASH domain of Nesprins. Canonical Nesprin isoforms have a variable sized N-terminus that projects into the cytoplasm and interacts with components of the cytoskeleton. This protocol describes the validation of a dominant-negative transgenic mouse strategy that disrupts endogenous SUN/KASH interactions in a cell-type specific manner. Our approach is based on the Cre/Lox system that bypasses many drawbacks such as perinatal lethality and cell nonautonomous phenotypes that are associated with germline models of LINC complex inactivation. For this reason, this model provides a useful tool to understand the role of LINC complexes during development and homeostasis in a wide array of tissues.

Video Link

The video component of this article can be found at http://www.jove.com/video/53318/

Introduction

The nuclear envelope (NE) separates the nucleoplasm from the cytoplasm. It is composed of an inner and outer nuclear membrane (INM and ONM, respectively) that connect at nuclear pores. The lumen delineated by both membranes is called the perinuclear space (PNS). The ONM is an extension of the rough endoplasmic reticulum (ER), and the INM adheres to the nuclear lamina, a meshwork of nuclear type-V intermediate filaments represented by A- and B-type lamins [1,2]. LInkers of the Nucleoskeleton and Cytoskeleton (LINC) complexes are macromolecular assemblies that span the whole nuclear envelope to physically connect the interior of the nucleus to cytoskeletal filaments and molecular motors (**Figure 1A**). They consist of interactions between evolutionarily conserved motifs that characterize two families of integral transmembrane proteins of the NE: Sun (Sad1/Unc84) proteins and Nesprins (Nuclear Envelope SPectRINS). In mammals, Sun1 and Sun2 are transmembrane proteins of the INM whose N-terminal nucleoplasmic region interacts directly with A- and B-type lamins [3-5]. On the other side of the INM, within the PNS, Sun proteins harbor an evolutionary-conserved stretch of ~150 C-terminal amino acids called the SUN domain. SUN domains interact directly with the evolutionary-conserved KASH (Klarsicht/Anc-1, Syne Homology) domain, the molecular signature of Nesprins. KASH domains consist of a stretch of ~30 C-terminal amino acids that protrudes into the PNS followed by a transmembrane domain [6]. At least four distinct Nesprin genes (Nesprin1-4) encode KASH-containing proteins that localize at the NE [7]. The cytoplasmic regions of Nesprins, whose sizes vary from ~50kDa (Nesprin4) to an astonishing 1,000 kDa (Nesprin1 giant), contain multiple spectrin repeats as well as specific motifs enabling their interaction with cytoskeletal components such as actin, plectin and molecular motors[8-13].

Studies in vertebrates and invertebrates have shown that Lamin/Sun/Nesprin/molecular motors constitute an evolutionarily conserved “axis” controlling nuclear migration and anchorage. Several knock-out (KO) mouse models of LINC complex components have been described and were instrumental in providing a framework to understand the roles of Sun and Nesprin proteins at the NE during mammalian development [9,14,15]. However, these models present several significant drawbacks, most notably: 1) difficulty in interpreting phenotypes due to cell non-autonomous effects, 2) difficulty in distinguishing the phenotypical contributions of KASH-containing vs. KASH-less Nesprin isoforms[16], 3) the functional redundancy of Sun and Nesprin proteins at the NE in numerous cell types requires complex breeding schemes to inactivate all SUN-KASH interactions in mice [17] and 4) the perinatal lethality of mice deficient for the KASH-domain of both Nesprins1 and 2 precludes the analysis of adult phenotypes [18].

This protocol describes a novel mouse model designed to disrupt all SUN-KASH interactions *in vivo*, in a cell autonomous and developmentally regulated manner, thus bypassing many of the drawbacks outlined above. This Cre/lox-based mouse model relies on two important concepts: 1) the KASH domain of any known Nesprin protein is sufficient to target EGFP to the NE in cell culture systems and 2) SUN domains interact promiscuously with KASH domains, thus overexpression of any KASH domain will saturate all endogenous SUN domains and inactivate LINC

complexes in a dominant-negative manner [17] (**Figure 1B**). This protocol describes tissue harvesting and processing steps used to confirm the disruption of all SUN-KASH interactions in cerebellar Purkinje cells.

Protocol

Ethics Statement: Procedures involving animal subjects were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University in St. Louis.

1. Mouse Breeding and Genotyping

1. Breed Tg(CAG-LacZ/EGFP-KASH2) mice with Tg(PCP2-Cre) mice to produce Tg(^{PCP2Cre}CAG-EGFP/KASH2) [19,20]. Note: While the remainder of this protocol focuses on the use of the Tg(PCP2-Cre) mouse line to restrict EGFP-KASH2 expression to Purkinje cells within the cerebellum, a similar procedure can be followed with any other Tg(Cre) mouse (**Figure 2A**).
2. Perform genotyping to identify pups harboring both EGFP and Cre transgenes as previously described [19].

2. Preparation of Materials for Dissection and Tissue Collection

1. Prepare 100 ml of 30% sucrose in PBS (30 g sucrose/100 ml of 1x PBS) and store at -20°C. The day of tissue harvesting, thaw and load ~30-35 ml of sucrose solution into a 35 ml syringe for transcardial perfusions.
2. Prepare 40 ml of 4% PFA in PBS (10 ml 16% paraformaldehyde and 30 ml PBS). Load ~30-35 ml of 4% PFA into a 35 ml syringe for transcardial perfusions.
3. Fill a 10 cm tissue culture plate with 10 ml of PBS for tissue dissection.
4. Prepare a ketamine/xylazine cocktail consisting of 16 mg/ml ketamine and 3 mg/ml xylazine.
5. Prepare a solution of 70% ethanol for the dissection.

3. Dissection and Tissue Collection

1. Using a 1 ml syringe, inject 500 µl of the ketamine/xylazine cocktail per 25 grams of body weight and wait for ~15 min. Alternatively, use another institutionally approved sedation method that is appropriate for the biological system of interest.
2. Upon sedation, gently pinch the rear paw to confirm the lack of pain response, pin all paws on a Styrofoam tray with the dorsal side of the mouse facing down.
3. Spray the abdomen with 70% ethanol and make an incision from the lower abdomen to the top of the rib cage to gain access to the heart.
4. Make a small incision in the right atrium of the heart and insert the syringe containing the 30% sucrose solution into the left ventricle of the heart.
5. Perfuse the solution at a rate of ~2-5 ml per min. When empty, remove the syringe and repeat with the 4% PFA solution.
6. Unpin the mouse from the Styrofoam tray and decapitate to access the brain.
7. Remove the skin around the head of the animal using forceps and scissors. Poke a hole in the posterior portion of the skull with a needle and carefully remove the skull with scissors and dissecting forceps.
8. Once the brain is clearly accessible, use forceps to remove the cerebellum and place it in PBS. Bisect the cerebellum in the sagittal plane with a scalpel and transfer to the 30% sucrose solution overnight at 4°C. Note: To prepare paraffin sections, fix the cerebellum overnight at 4°C in 4% PFA prior to tissue embedding and sectioning.

4. Tissue Processing and Sectioning

1. Prepare a slurry of dry ice and 2-methyl butane. Allow the temperature of the bath to equilibrate for 5 min.
2. Fill a cryomold with OCT compound and place one half of the bisected cerebellum into the mold.
3. Using a dissecting microscope and a pair of forceps position the cerebellum so that the bisected surface (near the midline of the cerebellum) is facing up.
4. Transfer the cryomold to the dry ice slurry for 5 min until the OCT compound has formed a solid white matrix around the tissue specimen. Note: The frozen specimen can now be stored at -20°C if required.
5. Label pre-treated poly-lysine slides for collecting tissue sections and prepare the cryostat according to the manufacturer's instructions in order to collect 15 µm sections of the cerebellum. To collect the sections, carefully contact the OCT section with the poly-lysine coated side of the slide. To preserve the orientation of the section, do not rotate the slide during this step.
6. Store tissue sections at -20°C until proceeding with staining and imaging.

5. Staining and Imaging of Cerebellum Sections

1. Prepare the following solutions prior to tissue staining:
   ~50 ml of PBS
   ~5-10 ml of 4% PFA in PBS
   ~10-20 ml of 0.5% Triton X-100, 10% donkey serum in PBS
2. Thaw cerebellar sections. Importantly, minimize their exposure to any source of continuous and intense light to prevent fading of EGFP.
3. Using a hydrophobic barrier pen, draw a rectangle around the tissue sections.
4. Post-fix the sections with 4% PFA for 5 min. Use enough liquid to cover the entire area delineated by the hydrophobic barrier (usually ~500 µl). Using a vacuum or pipette, remove the fixative being particularly careful not to disturb the tissue sections.

5. With a pipette, gently apply PBS on the slide without disturbing the tissue sections. After 5 min use the vacuum or pipette to carefully remove the liquid. Repeat the PBS rinse a total of three times.
6. Using a pipette, carefully apply the appropriate dilution of primary antibodies (3 µl Calbindin and/or Nesprin2 antibody per 900 µl of solution) in 0.5% Triton X-100, 10% donkey serum in PBS. Incubate for one hour at room temperature in the dark.
   Note: EGFP-KASH2 will be visible by direct fluorescence microscopy on frozen sections, and it is not necessary to utilize an anti-EGFP antibody. However, if paraffin sections have been prepared, imaging of EGFP-KASH2 requires immunodetection with an anti-EGFP antibody.
7. Remove the antibody solution and rinse with PBS three times.
8. Prepare 1:1,000 dilutions of Alexa-Fluor secondary antibodies in 0.5% Triton X-100, 10% donkey serum in PBS and store in the dark.
9. Gently remove the final PBS rinse and apply the secondary antibody dilution for one hour in the dark.
10. Remove the secondary antibody solution, rinse and wash the section three times. If needed, apply a nuclear stain, such as DAPI (~300nM), during the first PBS application.
11. Completely remove the PBS solution from around the tissue and place ~5-7 µl of mounting media on the tissue section. Place a glass coverslip on top of the tissue section and set aside for ~15-30 min at room temperature in the dark.
12. Use a fluorescence microscope equipped with the appropriate filters to visualize EGFP and any other wavelengths appropriate for the secondary antibodies used in steps 5.8/5.9. In cerebellar slices, use a 20X objective to view EGFP-KASH2 nuclear rims.

Representative Results

This protocol illustrates the usefulness of the Tg(CAG-LacZ/EGFP-KASH2) mouse model to restrict EGFP-KASH2 expression to cerebellar Purkinje cells using Tg(PCP2-Cre) mice. In Tg(^{PCP2Cre}CAG-EGFP/KASH2) offspring, the LacZ/V5 open reading frame is excised at P6 by the Cre recombinase thereby leading to the expression of EGFP-KASH2 specifically targeted to Purkinje cells (**Figure 2A**). As expected, EGFP-KASH2 is targeted to the nuclear envelope as indicated by the EGFP-positive rim-like pattern observed around the nucleus (**Figure 2B**). To maximize any physiological phenotype, it is important to ensure that EGFP-KASH2 is expressed in a majority of targeted cells by calculating the percentage of EGFP-KASH2 positive cells that are co-stained with a cell-specific antibody marker. In this example, EGFP-KASH2 expression was detected in more than 70% of Calbindin-positive Purkinje cells (**Figure 2B**). In these conditions, we did not observe any significant histological or behavioral phenotypes in 10 month-old Tg(^{PCP2Cre}CAG-EGFP/KASH2) mice[20]. It is important to ensure that any morphological or behavioral phenotype is not due to ectopic expression of EGFP-KASH2 in an unintended cell type/tissue. To that respect, EGFP-KASH2 was not observed in the granule cell layer or the molecular layer of Tg(^{PCP2Cre}CAG-EGFP-KASH2) cerebella (**Figure 2B**).

Finally, it is vital to confirm the extent of LINC complex disruption in cells expressing EGFP-KASH2. This can be achieved using high-quality anti-Nesprin antibodies to emphasize their displacement from the NE of cells expressing EGFP-KASH2. As shown in **Figure 3**, endogenous Nesprin2 is displaced from the nuclear envelope of Purkinje cells that express EGFP-KASH2 (**Figure 3** top panel, yellow arrows) while Purkinje cells that do not express EGFP-KASH2 retain endogenous Nesprin2 at the nuclear envelope (**Figure 3** top panel, white arrow). As expected, control littermates, which do not express Cre-recombinase, display Nesprin2 at the nuclear envelope of the whole Purkinje cell population (**Figure 3** bottom white arrows). Several reports have now confirmed the expression of EGFP-KASH2, accompanied by the displacement of endogenous Nesprins, in additional tissues and cell types such as skeletal muscle fibers and cone photoreceptors[19,21].

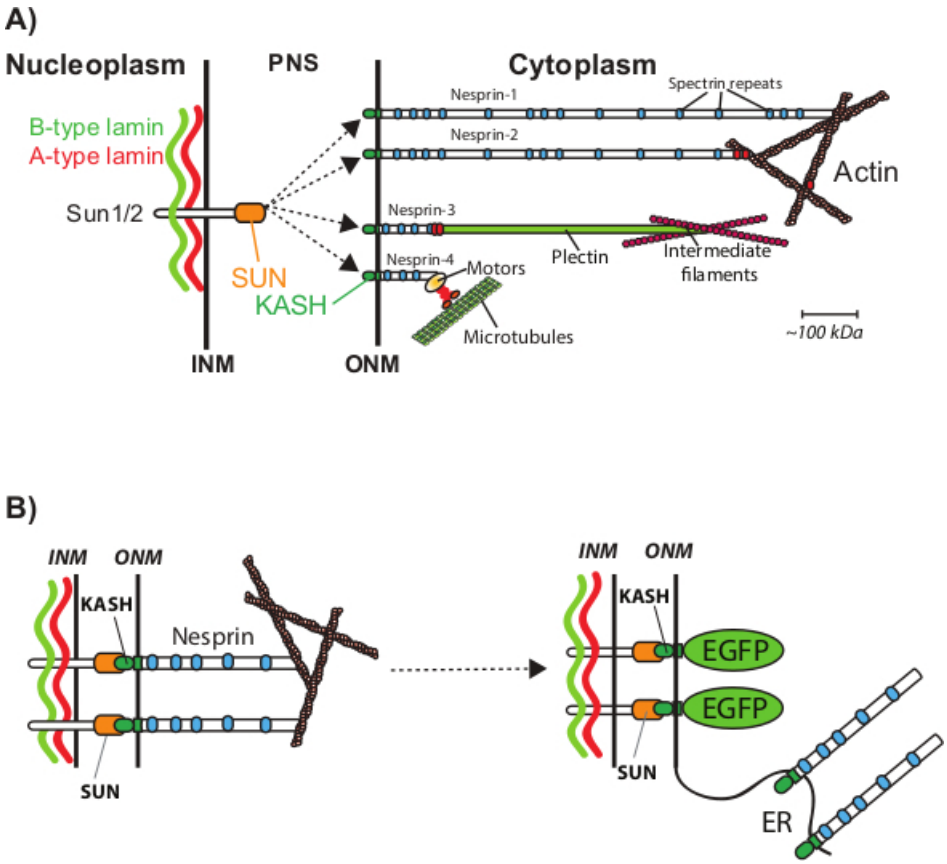

Figure 1. LINC complexes connect the nucleus to the cytoskeleton and are disrupted by the overexpression of EGFP-KASH2. (**A**) Depiction of LINC complexes that connect the interior of the nucleus to the cytoplasmic cytoskeleton. Note that SUN domains interact promiscuously with KASH domains from all Nesprins (Nesprins 1-4). (**B**) Overexpression of EGFP-KASH2 displaces endogenous Nesprins from the nuclear envelope to the ER, in a dominant negative fashion, thus disconnecting the nucleus from the surrounding cytoplasmic cytoskeleton.

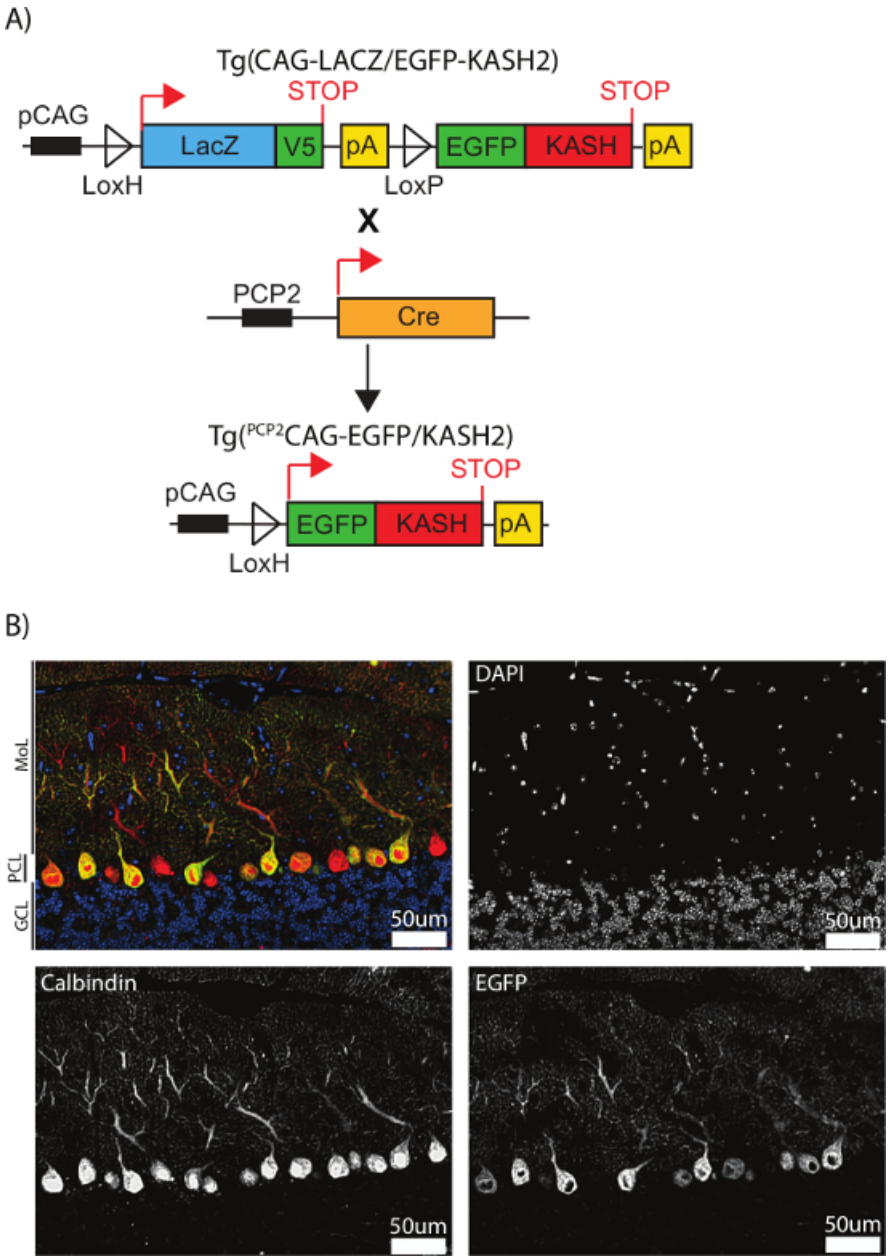

Figure 2. Overexpression of EGFP-KASH2 specifically targeted to cerebellar Purkinje cells. (**A**) Breeding of Tg(CAG-LacZ/EGFP-KASH2) to Tg(PCP2-Cre) mice results in Tg(^{PCP2Cre}CAG-EGFP-KASH2) offspring in which Cre recombinase is expressed specifically in cerebellar Purkinje cells. Upon expression (P6), Cre recombinase translocates into the nucleus where it excises the LacZ ORF and induces EGFP-KASH2 expression. (**B**) Tg(^{PCP2Cre}CAG-EGFP-KASH2) mice specifically express EGFP-KASH2 in cerebellar Purkinje cells identified with Calbindin (colored in red). Note that both cerebellar granule neurons and interneurons of the molecular layer are negative for EGFP-KASH2 expression. Abbreviations: MoL: Molecular layer, PCL: Purkinje cell layer and GCL: Granule cell layer.

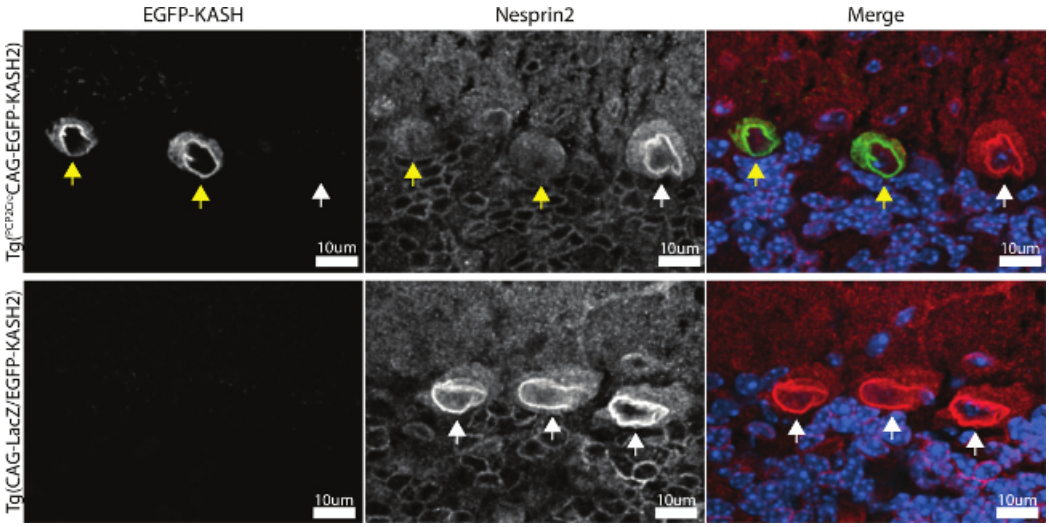

Figure 3. Disruption of endogenous LINC complexes in cerebellar Purkinje cells. Single confocal plane showing expression of EGFP-KASH2 that displaces endogenous Nesprin2 (colored in red) from the nuclear envelope of Purkinje cells (yellow arrows top). Note that Purkinje cells lacking EGFP-KASH2 expression maintain endogenous Nesprin2 at their nuclear envelope (white arrow top). In Tg(CAG-LacZ/EGFP-KASH2) control littermates, no EGFP signal is observed and Nesprin2 is detected at the nuclear envelope of all Purki

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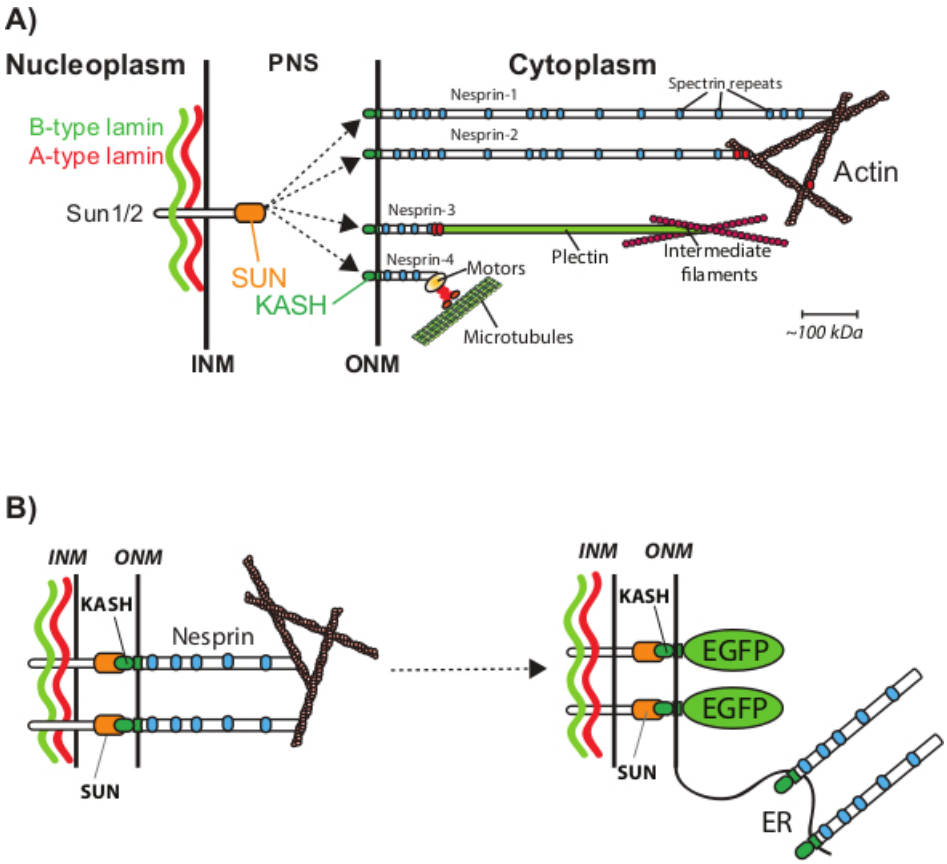

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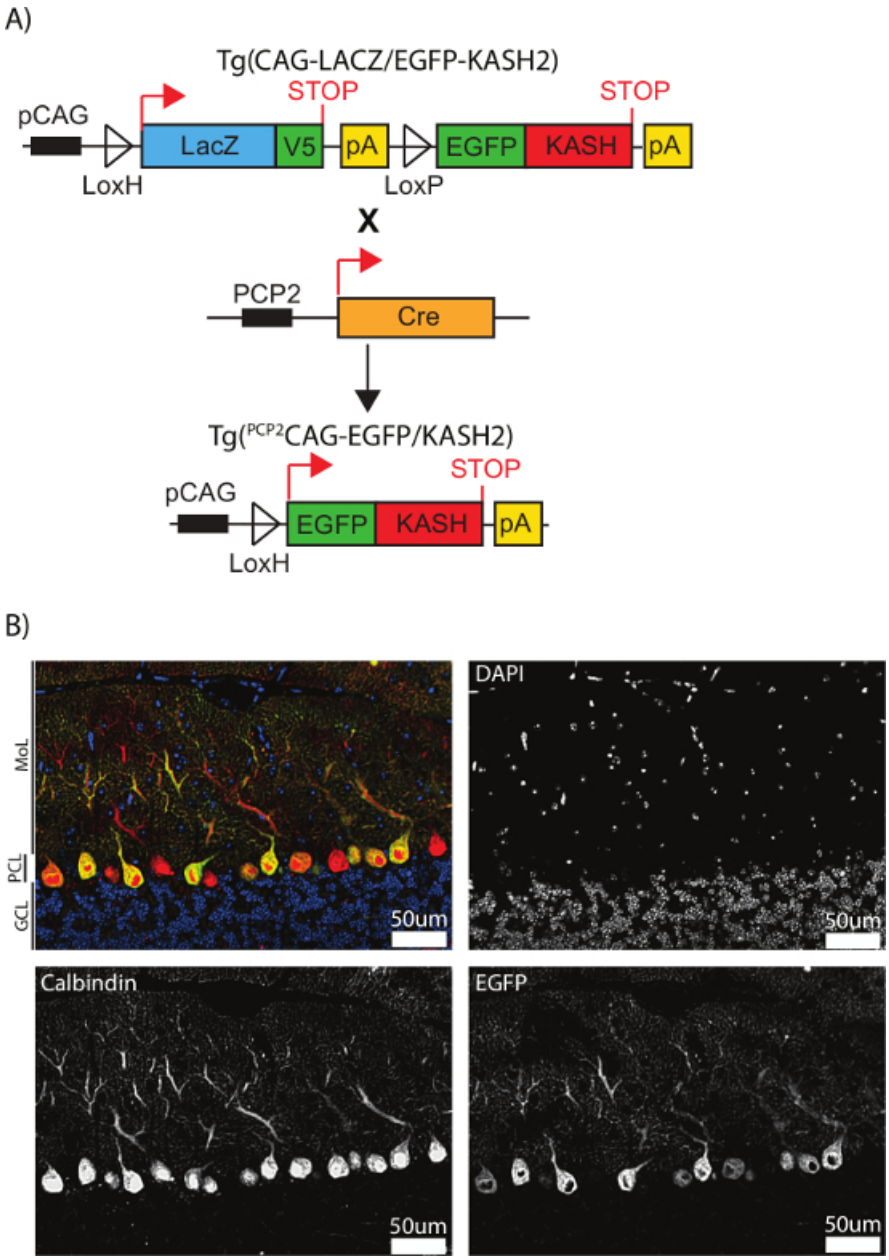

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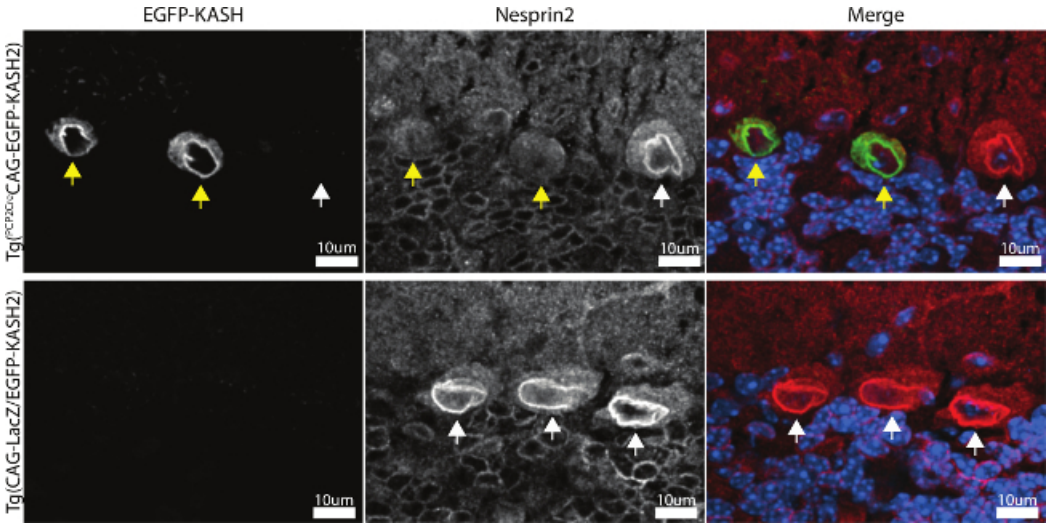

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Discussion

The most critical step to successfully study the role of LINC complexes *in vivo* using the Tg(CAG-LacZ/EGFP-KASH2) model is to identify a suitable Cre mouse line(s). Indeed, if Cre is active in other cell-types involved in similar pathways, it can complicate the interpretation of the results. Hence, it is important to examine nearby cells, as shown here in the molecular and granule cell layers of the cerebellum (**Figure 2B**). Likewise, to maximize any physiologically relevant phenotypes it is important to identify a Cre strain with widespread Cre expression across the cell type of interest. It is also vital to undertake an in-depth characterization of the expression pattern of LINC complex components during the development of the system of interest. Indeed, one drawback of the Tg(CAG-LacZ/EGFP-KASH2) mouse model is that it does not provide any information as to which Sun proteins or Nesprins variants are involved in the biological system under consideration. However, this issue can be overcome by undertaking a rigorous characterization at both the transcript and protein levels. In fact, such studies have been undertaken on several occasions which led to important findings about the role of specific LINC complex components during CNS development and homeostasis[21,22].

Finally, while this protocol is focused on the use of fixed CNS tissues, Tg(CAG-LacZ/EGFP-KASH2) mice can be used to isolate and culture primary cells to study the effect of disrupting LINC complexes *in vitro*. Using this scheme, it is possible to isolate and purify primary cultures of EGFP-KASH2-positive cells for applications such as time-lapse video microscopy. This bypasses low transfection efficiencies or lentiviral production required for primary culture systems, such as neurons.

In summary, the Tg(CAG-LacZ/EGFP-KASH2) mice open up new and exciting possibilities to study the function of LINC complexes *in vivo* during embryonic and postnatal mammalian developmental processes in a wide variety of tissues and cell types to address basic cell biology questions.

Disclosures

The authors have nothing to disclose.

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