i. Chapter Title

Analysis of nuclear pore complexes in *Caenorhabditis elegans* by live imaging and functional genomics

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ii. Summary/Abstract

Nuclear pore complexes (NPCs) are essential to communication of macromolecules between the cell nucleus and the surrounding cytoplasm. RNA synthesized in the nucleus is exported through NPCs to function in the cytoplasm, whereas transcription factors and other proteins are selectively and actively imported. In addition, many NPC constituents, known as nuclear pore proteins (nucleoporins or nups), also play critical roles in other processes, such as genome organization, gene expression and kinetochore function. Thanks to its genetic amenability and transparent body, the nematode *Caenorhabditis elegans* is an attractive model to study NPC dynamics. We provide here an overview of available genome engineered strains and FLP/Frt-based tools to study tissue-specific functions of individual nucleoporins. We also present protocols for live imaging of fluorescently tagged nucleoporins in intact tissues of embryos, larvae and adult and for analysis of interactions between nucleoporins and chromatin by DamID.

iii. Key Words

Caenorhabditis elegans; CRISPR-Cas9; DamID; FLP; live imaging; npc; npp; nuclear pore complex; nucleocytoplasmic transport; nucleoporin.

1. Introduction

The nematode *Caenorhabditis elegans* has been a popular and powerful model organism for more than half a century thanks to pioneering work by Sydney Brenner [1]. The transparency allows live observation of complex processes, such as organ development and neuronal activity in the intact organism with non-invasive techniques. Cell divisions are stereotypic in space and time, producing an invariant cell lineage [2,3]. The development of *C. elegans* from egg through four larval stages (L1-L4) to fertile adult takes approximately three days. The adult hermaphrodite produces ~250 progeny over a time course of 3-5 days, and has a lifespan of 2-3 weeks. *C. elegans* embryos are surrounded by a resistant eggshell, which facilitates mounting for long time-lapse observation from the zygote to the fast-moving three-fold embryo ready to hatch. Larvae and adults are small (up to ~1 mm) which enables high-resolution live imaging of individuals that can be either anaesthetized or immobilized by microfluidics devices [4] (Figure 1). Despite their short lifespan, changes in nuclear envelope morphology are observed in old individuals, making *C. elegans* an attractive model to study aging [5]. *C. elegans* can easily be manipulated in large numbers and is thus very suitable for high-throughput forward and reverse genetic studies. This has revealed roles of *C. elegans* nucleoporins (nups; in *C. elegans* nomenclature known as Nuclear Pore Proteins or NPPs) in a variety of processes, such as germ granule distribution [6], transposon silencing [7], sensitivity to ionizing radiation [8], transposon silencing [7] and cell polarity [9]. Most nups are conserved in *C. elegans* (Table 1) and it is reasonable to assume that the three-dimensional structure of the NPC and its function in nucleocytoplasmic transport are also maintained [10,11]. Similarly to the situation in other organisms, *C. elegans* nups have been shown by chromatin immunoprecipitation (ChIP) and DNA adenine methylation identification (DamID) to interact with the genome, suggesting that certain nups might be directly involved in regulation of gene expression [12-14]. Interestingly, recent single cell analyses suggest that the ratio between individual *C. elegans* nups varies significantly between cell types although this remains to be confirmed at the protein level [15,11,16]. Moreover, it has been proposed that certain nups are expressed only during embryogenesis and larval development and remain stably integrated in NPCs during the entire lifespan of the animal [17].

Transgenes can be inserted efficiently into specific sites in the genome of *C. elegans* and CRISPR technologies allow precise point mutations, gene knockouts and insertion of short tags or genes encoding fluorescent proteins (Table 1) [18-20]. We have recently developed a versatile toolkit for FLP/Frt-mediated recombination in *C. elegans*, which enables spatiotemporal control of gene expression, cell ablation and conditional knockout (Figure 2 and Table 2) [21-23]. This has opened the possibility to perform tissue-specific DamID experiments to determine protein-DNA contacts in intestine and muscles [24,25]. Efficient systems for inducible and rapid protein degradation are also available [26,27] and the various tools can be further combined to increase their utility (Figure 2). We provide here a protocol for live imaging of embryos, larvae and adults, using strains with endogenously GFP-tagged nups as examples. Protocols for analysis of fixed embryos by immunofluorescence and electron microscopy are available elsewhere [28]. We also present details on how to identify chromatin domains bound by proteins of interest by DamID. Finally, we encourage the reader to consult excellent chapters on *C. elegans* biology and methods at WormBook (http://www.wormbook.org).

2. Materials

2.1 Live imaging

1. *C. elegans* strains expressing a protein(-s) of interest fused to a fluorescent protein(-s) (see Table 1).
2. Pipette and tips (10-1000 μL).
3. Glass slides (25 mm × 75 mm × 1 mm).
4. Label tape.
5. Cover slips (12 mm × 12 mm and 22 mm × 22 mm).
6. Worm pick with platinum wire.
7. Eyelash mounted on toothpick or Pasteur pipette.
8. 2% agarose solution in distilled water, melted in microwave oven and kept molten in a heat block at 65°C.
9. M9 buffer: 22 mM KH_2PO_4, 34 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$.
10. Meiosis buffer: 25 mM HEPES pH 7.4, inulin 0.5 mg/ml, Leibovitz L-15 medium 60%, fetal bovine serum 20%.

11. Levamisole stock solution: 100 mM tetramisole hydrochloride (e.g. Sigma-Aldrich L9756). Dilute 1:10 in M9 buffer to obtain 10 mM working solution.
12. VALAP: 1:1:1 mixture of Vaseline or petroleum jelly, lanolin, and paraffin. Melts at 60ºC.
13. Potassium phosphate 1M pH 6: 132 mM KH_2PO_4, 868 mM KH_2PO_4.
14. NGM plates (25 mM potassium phosphate pH 6, 51 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 17 g/l agar, 2.5 g/l peptone, 5 mg/l cholesterol) seeded with OP50 *Escherichia coli* bacteria (*see* Note 1).

2.2 DamID

1. *C. elegans* strains expressing *E. coli* Dam fused to either a protein of interest (test protein) or GFP (normalization control) (*see* Table 1 and Note 2).
2. NGM plates seeded with *E. coli* that does not express Dam, for instance GM119.
3. M9 buffer: 22 mM KH_2PO_4, 34 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$.
4. Hypochlorite solution: 1 N NaOH, 30 % household bleach solution.
5. DNeasy Blood and Tissue Kit (QIAGEN #69504).
6. RNAse A (Qiagen #19101).
7. *Dpn*I enzyme with 10x CutSmart buffer (NEB R0176S, 20 U/*µ*l).
8. T4 DNA ligase (Roche #10799009001, 5 U/*µ*L).
9. *Dpn*II enzyme with 10x*Dpn*II reaction buffer (NEB R0543S, 10 U/*µ*l).
10. Taq DNA Polymerase with ThermoPol Buffer (NEB M0267S, 5 U/*µ*l).
11. dNTP mix 2.5 mM (Takara SD0696)
12. Primer AdRt 5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA (100 *µ*M).
13. Primer AdRb 5'-TCCTCGGCCG (100 *µ*M).
14. AdR primers: combine equal volumes of:
    - 4N: 5'-NNNNGTCCTCGCGGCCGAGGATC (50 *µ*M)
    - 5N: 5'-NNNNNGTCCTCGCGGCCGAGGATC (50 *µ*M)
    - 6N: 5'-NNNNNNGTCCTCGCGGCCGAGGATC (50 *µ*M)
15. QIAquick® PCR Purification Kit (QIAGEN #28104).
16. NEBNext® Ultra DNA Library Prep Kit for Illumina® (NEB E7370).

17. NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1 E7335S; for up to 12 pooled samples. Combine with Index Primers Set 2 E7500S for up to 24 pooled samples).
18. SpeedBead Magnetic Carboxylate Modified Particles (GE Healthcare #65152105050250).
19. Ethanol 100% as well as freshly prepared 70% and 80% (store at -20ºC).
20. Sodium acetate 3M (pH 5.2).
21. QUBIT® fluorometer and dsDNA HS Assay Kit (Invitrogen Q32854).
22. Tubes suitable for QUBIT (e.g. DNA LoBind Tube 0.5 ml; Eppendorf #0030108035)
23. Thermocycler.
24. Magnetic particle concentrator (e.g. DynaMag-PCR; Invitrogen #492025).
25. Glycogen 20 mg/ml (Roche #10901393001).

3. Methods

3.1 Live imaging of whole animals

Live imaging requires both restriction of movement during image acquisition and preservation of cell and tissue integrity. If a developmental process is to be studied for tens of minutes or even hours, particular care has to be devoted to establishing mounting conditions that do not interfere with the process, nor cause photo toxicity or bleaching by sample illumination (*see* Note 3).

1. Place two glass slides labelled with tape in parallel separated by a third clean microscope slide on a flat surface.
2. Using a pipette, place approximately a 50 μL drop of 2% melted agarose onto the center of the clean slide.
3. Instantly, cover the agarose drop with another glass slide in perpendicular way to make agarose flat before it solidifies. Avoid bubbles in the agarose to ensure a flat, intact pad (*see* Note 4).
4. Once agarose is solidified, move away the labelled slides and detach the two remaining slides carefully so that the agarose pad stays on one of them. Place the slide on the bench with the agarose facing upwards.

5. Place a 3 μl drop of 10 mM levamisole onto the center of the agarose pad (*see* Note 5).
6. Transfer 2-3 animals using a worm pick into the drop where they will float off. To avoid transfer of excess bacteria, pick worms from a bacteria-free zone of the plate or transfer worms to a plate without bacteria before making the agarose pad. Relocate the animals in the desired positions with the eyelash while the drop progressively dries up around them. Immediately place the coverslip before the drop disappears and refill the space under the coverslip with M9 buffer to avoid desiccation of the animals (*see* Note 6).
7. Seal the coverslip with melted VALAP. This is most easily done with a fine paintbrush.
8. Acquire microscopy images at 20-24ºC within 30 minutes after mounting the sample (*see* Note 7).
9. Recover the animals from the agarose pad if they are to be analyzed again later. Gently remove the coverslip while observing the animals in a stereoscope. Add 5 μl of M9 buffer and transfer the animals to a 10 μl drop of M9 on an NGM plate seeded with OP50 bacteria (*see* Note 1).

3.2 Live imaging of embryos

The *C. elegans* embryo is protected by a robust eggshell and can easily be prepared for live imaging. However, it requires some practice to obtain early 1-cell stage embryos (*see* Note 8).

1. Prepare a slide with a 2% agarose pad as described in steps 1-4 above (Section 2.1).
2. Transfer 2 young adult hermaphrodites to a 3 µl drop of M9 buffer on a 12-mm square glass coverslip (*see* Notes 9-10). Selection of L4 stage animals the day before imaging ensures having semi-synchronized young adults.
3. Use 2 syringe needles (e.g. 25-26 GA) to cut the animals in the middle to release the embryos from the uterus.
4. Lower gently the agarose pad (upside down) until it contacts the drop containing the embryos. The coverslip will adhere to the agarose pad.

5. Optionally, seal the coverslip using melted VALAP if the recording is planned to last for long time (more than 40 min).
6. Using a low-magnification objective, quickly select a suitable embryo and switch to a 60X or 100X oil objective for recording with the best possible image resolution using fluorescent microscopy combined with DIC imaging (*see* Notes 11-14).

3.3 DamID

The protocol presented here is optimized for using ~4000 nematodes per genotype and replica (*see* Note 15).

1. Collect embryos from asynchronous cultures by standard hypochlorite treatment (*see* Notes 16-17).
2. Leave embryos to hatch overnight at 16–20°C in M9 with gentle agitation (~120 rpm).
3. Determine the number of hatched L1 larvae and unhatched embryos in 2–10 µL aliquots. The hatching rate should be higher than 70%.
4. Aliquot ~1000 L1s onto 100 mm NGM plates containing Dam-negative *E. coli* as food source (*see* Note 18).
5. Incubate nematodes at a suitable temperature for your experiment until they reach the desired life stage and collect them with M9 in a conical 15 ml tube.
6. Wash the nematodes 10 times with 15 ml of M9. Pellet them by centrifugation at 800g for 1 minute in each wash. Extensive washes are essential to avoid *E. coli* sequences in the final library.
7. Perform 5 rounds of freezing/thawing in liquid nitrogen and a 37ºC water bath or thermoblock to break the animals' cuticle.
8. Purify genomic DNA using the DNeasy Blood and Tissue Kit (including the optional RNase treatment step). Handle the DNA with care to avoid shearing, which can lead to amplification of unspecific sequences at later steps in the protocol. Elute the DNA in two steps: first in 100 µl and next in 200 µl. Measure DNA concentration using QUBIT. Mix both eluates if needed and precipitate the DNA if the concentration is < 25 ng/µl (*see* Note 19).

9. Digest 200 ng DNA using 10 units *Dpn*I in 10 µl for 6 hours at 37°C in a thermocycler (*see* Note 20).
10. Heat-inactivate *Dpn*I at 80°C for 20 minutes.
11. Prepare double-stranded (ds) adaptors by mixing 50 µl primer AdRt and 50 µl primer AdRb to have a final concentration of 50 µM. Heat the mix to 95 °C during 10' and let the samples cool down slowly until room temperature is reached. Adaptors can be frozen at -20ºC.
12. Prepare ligation mix containing 6.2 µl ddH_2O, 0.8 µl ds AdRt+AdRb adapters, 2 µl 10x T4 DNA ligase buffer and 1 µl T4 DNA ligase per sample.
13. Add 10 µl of ligation mix to each 10 µl reaction from step 10 and incubate at 16 °C overnight in a thermocycler.
14. Heat-inactivate T4 DNA ligase at 65°C for 10 minutes.
15. To purify the DNA, vortex SpeedBeads and add x1.8 volume (36 µl) of resuspended beads to the reaction from step 14 (*see* Note 21). Mix well by pipetting up and down at least 10 times.
16. Incubate for 5 minutes at room temperature.
17. Place the tubes (open) on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (~5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads.
18. Add 180 µl of 80% ethanol to the tubes while in the magnetic stand (on the opposite wall where the beads are). Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant, without touching the beads on the wall of the tube. Discard the last part of the liquid with a P10 pipette.
19. Repeat step 18 once.
20. Air dry the beads for 5 minutes while the tubes are on the magnetic stand with the lid open. Do not overdry the beads as this may result in lower recovery of DNA.
21. Remove the tubes from the magnet. Elute DNA from beads into 22 µl elution buffer (EB from QIAquick PCR Purification Kit; Tris/HCl 10 mM pH 8.5). Mix well by pipetting up and down at least 10 times. Incubate at room temperature for 2 minutes.

22. Place the tube on the magnetic stand to separate beads from supernatant. After the solution is clear (~5 minutes), carefully transfer 20 µl supernatant to a 0.2 ml PCR tube.
23. Digest the purified DNA with 10 units of *Dpn*II in a volume of 50 µl for 1 h at 37°C in a thermocycler.
24. Inactivate *Dpn*II at 80ºC for 20 minutes.
25. Purify the reaction with SpeedBeads using 90 µl (1.8x volume) of resuspended beads to the 50 µl resulting from DpnI digestion. Mix well by pipetting up and down at least 10 times.
26. Incubate for 5 minutes at room temperature.
27. Place the tubes (open) on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (~5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads.
28. Add 180 µl of 80% ethanol to the tubes while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant, without touching the beads on the wall of the tube. Discard the last part of the liquid with a P10 pipette.
29. Repeat step 28 once.
30. Air dry the beads for 5 minutes while the tubes are on the magnetic stand with the lid open. Take care not to overdry the beads.
31. Remove the tubes from the magnet. Elute DNA from beads into 27 µl EB. Mix well by pipetting up and down at least 10 times. Incubate at room temperature for 2 minutes.
32. Place the tube on the magnetic stand to separate beads from supernatant. After the solution is clear (~5 minutes), carefully transfer 25 µl supernatant to a new PCR tube.
33. Prepare PCR mix containing 13.75 µl ddH_2O, 1.25 µl AdR primers (1:1:1 mix; final concentration 50 µM), 5 µl 10x ThermoPol buffer, 4 µl dNTP mixture (2.5 mM) and 1 µl Taq polymerase per sample.
34. Add 25 µl of the PCR mixture to the 25 µl reaction from step 33. PCR conditions: (1) 68°C for 10 minutes; (2) 94°C for 1 minute; 65°C for 5 minutes; 68°C for 15 minutes; (3) 94°C for 1 minute; 65°C for 1 minute; 68°C for 10 minutes; (4) repeat step (3) x2;

(5) 94°C for 1 minute; 65°C for 1 minute; 68°C for 2 minutes (6) repeat step (5) x14 (*see* Note 22).

35. Purify the PCR products using QIAquick PCR Purification Kit and elute in 30 μl of EB. Measure DNA concentration using QUBIT (*see* Note 23).
36. Run 1-2 μl of the PCR products on a chip-based capillary electrophoresis system (e.g. Agilent Bioanalyzer). Successful amplification of Dam-methylated genomic DNA should yield a smooth distribution of 400-1000 bp fragments except in the negative controls (*see* Note 17).
37. For library preparation, mix 50 ng of PCR product with 3.0 μl NEBNext End Prep Enzyme Mix (from E7370) and 6.5 μl 10x NEBNext End Repair Reaction Buffer (from E7370) in a total volume of 65 μl.
38. Incubate in a thermocycler for 30 minutes at 20°C followed by 30 minutes at 65°C.
39. For adapter ligation, add the following components directly to the End Prep reaction mixture and mix gently: 15 μl Blunt/TA Ligase Master Mix (from E7370), 2.5 μl NEBNext Adaptor for Illumina (from E7335/E7500) and 1 μl Ligation Enhancer (from E7370).
40. Incubate in a thermocycler for 15 minutes at 20°C.
41. Add 3 μl of USER Enzyme (from E7335/E7500) to the ligation mixture from the previous step and mix gently.
42. Incubate in a thermocycler for 15 minutes at 37°C.
43. Add 13.5 μl ddH_2O to reach a total volume of 100 μl.
44. Purify and size-select the reaction by adding 30 μl (0.3x volume) of resuspended SpeedBeads and mix well by pipetting up and down at least 10 times (*see* Note 24).
45. Incubate for 5 minutes at room temperature.
46. Place the tubes on a magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant to new tubes. Discard the beads that contain unwanted large DNA fragments.
47. Add 74 μl (0.8x volume) resuspended SpeedBeads to the supernatant, mix well and incubate for 5 minutes at room temperature.

48. Place the tubes on the magnetic stand to separate the beads from the supernatant. After the solution is clear (~5 minutes), carefully remove and discard the supernatants that contains unwanted small DNA fragments. Be careful not to disturb the beads.
49. Add 180 μl of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
50. Repeat step 49 once.
51. Air dry the beads for 5 minutes while the tubes are on the magnetic stand with the lid open. Take care not to overdry the beads.
52. Remove the tubes from the magnetic stand. Elute the DNA target with 17 μl of EB. Mix well on by pipetting up and down. Incubate for 2 minutes at room temperature.
53. Place the tubes again on the magnetic stand. After the solution is clear (~5 minutes), transfer 15 μl to a new PCR tube.
54. For PCR enrichment of adaptor ligated DNA, add 25 μl NEBNext Q5 Hot Start HiFi PCR Master Mix (from E7370), 5 μl Universal PCR Primer (from E7335/E7500) and 5 μl sample-specific Index Primer to the 15 μl reaction from step 53 (*see* Notes 25-26).
55. PCR cycling conditions: (1) 98ºC for 30 seconds; (2) 98ºC for 10 seconds; (3) 65ºC for 75 seconds; (4) repeat (2)-(3) x7; (5) 65ºC for 5 minutes.
56. For purification of the PCR amplicons, add 90 μl (1.8x volume) of resuspended Speed-Beads to each PCR tube. Mix well by pipetting up and down and incubate for 5 minutes at room temperature.
57. Place the tubes on the magnetic stand to separate the beads from the supernatant. After the solution is clear (~5 minutes), carefully remove and discard the supernatants that contains unwanted DNA. Be careful not to disturb the beads.
58. Add 180 μl of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
59. Repeat Step 58 once.
60. Air dry the beads for 5 minutes while the tubes are on the magnetic stand with the lid open. Take care not to overdry the beads.

61. Remove the tubes from the magnetic stand. Elute the DNA target with 33 µl of EB. Mix well on by pipetting up and down. Incubate for 2 minutes at room temperature.
62. Place the tubes again on the magnetic stand. After the solution is clear (about 5 minutes), transfer 28 µl to a new tube. Libraries can be stored at -20ºC.
63. Run 3 µl of each sample on a 1% agarose gel to check that the 400-1000 bp size distribution observed in step 18 is maintained. Measure DNA concentration using QUBIT (*See* Note 27).
64. Prepare the library for sequencing by pooling samples according to the instructions indicated by the sequencing facility, including an optional re-purification of the library with 1x volume SpeedBeads (*See* Note 28).

Processing and analysis of DamID data go beyond the scope of this chapter. Instead, we refer the readers to step-wise and all-in-one pipelines that have been described for *C. elegans* and Drosophila data analysis [29-32].

4. Notes

1. NGM plates are prepared according to standard *C. elegans* protocols [33].
2. To generate DamID strains expressing proteins of interest fused to Dam, we refer to recent protocols, which include cloning, transgenesis and validation steps [29,14].
3. For time lapse recordings, we recommend lower concentration of anaesthetizing reagents. For instance, for *in utero* imaging of oocytes and newly fertilized embryos during 40-60 minutes, young adult hermaphrodites can be anesthetized in 20 µL 5 mM tricaine (ethyl 3-aminobenzoate methanesulfonate), 0.5 mM levamisole, 0.5x M9 for 15–20 minutes prior to mounting in 3 µL of the same buffer on 2% agarose pads [34]. For longer recordings, animals can be immobilized up to 5–6 hours by incubation for 30 minutes in 1.67 mM tricaine, 167 µM levamisole, 0.5x M9 before mounting in 3 µL of the same buffer on 3% agarose pads [35]. Keep in mind that the response to levamisole changes during development and aging, which implies that further optimization might be needed for particular experiments. Finally, it is

also relevant to point out that levamisole can induce hypercontraction of the pharyngeal muscle, and thereby affect morphology of the anterior part of the animal.

4. To avoid formation of bubbles in the agarose pad it is important to release the agarose drop slowly into the glass slide and gently press the upper slip to adjust the thickness of the agarose layer. The pipette tips can be cut at the end to facilitate the release of the agarose.
5. If faster and more complete immobilization is needed, 3 mM sodium azide (NaN_3) can be added to the 10 mM levamisole solution. However, this will reduce the possibility to recover the animals alive. If the particular strain to be analyzed is resistant to levamisole or if the drug might affect the process of interest, polystyrene nanoparticles can be used as an alternative immobilization method [36].
6. To avoid sample desiccation, it is crucial to pay attention when liquid around the animals is vanishing (due to evaporation and absorption into the agarose pad) and place immediately the coverslip.
7. We recommend confocal microscopy. Images in Figure 1A-B were acquired with a Nikon Eclipse Ti microscope equipped with Plan Fluor 40x/1.3 and Plan Apo VC 60x/1.4 objectives and a A1R scanner using a pinhole of 1.2–1.4 airy unit. Acquisition of z-stack images was performed with integrated Nikon NIS software. Image stacks were processed with Fiji/ImageJ [37].
8. The eggshell matures ~30 minutes after fertilization, which implies that the earliest 1-cell stage embryos are more sensitive to osmotic and physical pressure. These embryos can be mounted in meiosis buffer instead of M9 buffer or observed *in uterus* as described in Note 3.
9. Optionally, using M9 containing 10 μM levamisole facilitates extrusion of embryos during dissection.
10. To select and mount multiple embryos of a desired stage, dissect 4-6 adults in M9 buffer in a watch glass. Disperse the embryos with the dissection needles and identify the relevant embryos in a high-magnification stereoscope. Transfer the

embryos with an ultrafine pipette to an agarose pad. Use an eyelash to position the embryos on the pad as described in section 3.1 step 6 above.

11. If using an inverted microscope, application of oil to the high-magnification objective can be challenging. If possible, mark the position of the relevant embryo(-s) in the microscope software when observing with the low-magnification objective. Move the stage so objectives can be accessed and change to the high-magnification objective. Apply oil and lower the objective before using the software to return to the position of the embryo(-s).
12. Imaging conditions will depend on the particular microscope, intensity and photostability of the fluorescent proteins expressed in the embryo. Often one has to find a compromise between illumination intensity, interval between image acquisition and total duration of recording. Processes in the early embryos are typically so fast that image acquisition of multiple z positions require very rapid microscopes, such as spinning disk systems. Otherwise, subcellular structures will have moved considerably during acquisition from the first to the last z position. We typically aim for single confocal images every 5-12 seconds on point scanning confocal microscopes or z stacks of ~17 focal planes (~5 μm) every 12 seconds on spinning disk confocal microscopes.
13. Embryos can be recorded at temperatures between 16ºC and 25ºC. Higher temperatures negatively affect embryogenesis and the fact that the temperature of the samples can easily be 1-2 ºC higher than the room temperature due to heating of the microscope and objective should be taken into account. For optimum temperature control during live imaging, including precise cold and heat shock treatments, we recommend the fast-acting CherryTemp temperature controller from Cherry Biotech (see e.g. [38]) or alternative systems [39]. In addition, even subcellular temperature control can be achieved with FLIRT (fast local infrared thermogenetics) microscopy [40].
14. Simultaneous acquisition of high-quality differential interference contrast (DIC) images is advantageous, as it offers the possibility to track unlabeled structures, such as centrosomes, yolk granules, plasma membrane fluctuations, etc.

15. An alternative DamID protocol for as little as 20 adults is available [13].
16. For synchronization of nematodes by hypochlorite treatment ("bleaching"), a mixed-stage population with many gravid adults is washed off an NGM plate with M9 into a 15 ml tube and centrifuged at 500 g during 2 minutes. Aspirate the supernatant and resuspend the animals in 15 ml M9 buffer and centrifuge again at 500 g for 2 minutes. Repeat the washing and aspirate as much as supernatant as possible without disturbing the pellet of worms. Add 2 ml of hypochlorite solution and shake. Monitor the animals under a stereoscope until the majority are broken up (depends on the concentration of the hypochlorite; should not be more than 5-6 minutes) and fill immediately the tubes with sterile M9. Centrifuge at 800 g for 1 minute. Aspirate the supernatant and resuspend the embryos in 15 ml M9 buffer and centrifuge again at 800 g for 1 minute. Repeat the washes 10 times (in sterile conditions) and let in a suitable volume of M9 to reach ~20 embryos/µl.
17. Include a strain that does not express Dam fusion proteins, for instance the standard N2 reference strain, as negative control during steps 1-35. Alternatively, prepare a control without *Dpn*I enzyme (step 9) and a control without T4 DNA ligase (step 13) with animals that express the DamID constructs.
18. Using Dam-negative *E. coli* as a food source is important to avoid contamination of the NGS library by methylated *E. coli* DNA. Growing the worms for a couple of generations on Dam-negative plates *E. coli* prior to the experiment is recommended. The Dam-negative *E. coli* plates should not be older than 1 week.
19. To precipitate the DNA, add 1 µl 20 mg/ml glycogen and mix gently. Next, add 30 µl 3M sodium acetate and 750 µl ice cold 100% ethanol. Incubate at -80ºC for 1 hour or longer. Centrifuge at ~16,000 g, 4ºC for 30 minutes. Carefully remove the supernatant and add 180 µl ice cold 70% ethanol. Centrifuge immediately at ~16,000 g, 4ºC for 10 minutes. Carefully remove the supernatant, spin briefly, remove any remaining supernatant and leave to air dry for 10 minutes. Resuspend gently with 10 µl of elution buffer from QIAquick PCR Purification Kit (Tris/HCl 10 mM pH 8.5). Leave at 4ºC overnight before measuring the concentration with QUBIT.

20. Lower (or higher) amounts of genomic DNA can be used. In this case, adjust the number of PCR cycles in step 34.
21. We recommend testing the performance of the SpeedBeads, for instance by purification of a 100 bp DNA ladder. Size selection should deplete fragments smaller than 400 bp.
22. The number of PCR cycles should be optimized in a pilot experiment. Prepare 50 µl reactions for 1-2 samples and a negative control. Take out 10 µl for chip-based capillary electrophoresis after 12, 14, 16, 18 and 20 cycles. Depending on the electrophoresis system, purification of the PCR products (e.g magnetic beads or columns) might be required. Choose the lowest number of cycles that produce a smooth distribution of 400-1000 bp fragments in the samples but not in the negative control. Repeat this optimization step when using a new batch of Taq polymerase.
23. Expected yield is 2-20 ng/µl. You can stop and freeze (-20ºC) this step. The controls described in Note 17 are not included in the next steps of the protocol.
24. Vortex the Speed-Beads before use to ensure they are fully resuspended. The performance of the Speed-Beads should be tested monthly using a standard DNA ladder.
25. Consult the NEB E7335 and E7500 manuals for optimal combinations of index primers.
26. A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while performing size selection/cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.
27. Expected yield is 15-40 ng/µl.
28. We found that an additional purification of the pooled library with SpeedBeads improves the sequencing run. This can be performed as described in section 3.3 steps 56-62 but with a lower amount of SpeedBeads (1x volume) and eluting in a final volume of 15-30 µl.

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Figure Captions

Figure 1. Live imaging of endogenously tagged nucleoporins. (A) Schematic representation of the NPC. Nucleoporins are categorized based on their localization and form in several cases biochemically stable subcomplexes. Modified from [10]. (B) Expression levels of GFP-tagged MEL-28/ELYS, NPP-21/TPR and NPP-24/NUP88 in hypodermal cells of L1 larvae were compared by confocal live microscopy. NPP-21/TPR is the most abundant of the three, followed by NPP-24/NUP88. Scale bar 10 um. (B) During aging, the accumulation of MEL-28/ELYS at NPCs is diminished compared to the nuclear interior. (C) *C. elegans* is suitable for whole-animal imaging by live SPIM microscopy. In this example NPP-21/TPR::GFP was observed on a MuVi Luxendo Light-Sheet Microscope. The magenta signal in (A) and (C) represents mCherry-tagged histone H2B (HIS-58).

Figure 2. Strategies for analysis of *C. elegans* nups by FLP/Frt-mediated genome recombination. (A) Insertion of a GFP cassette containing Frt sites in introns 1 and 2 of GFP immediately upstream of a gene of interest serves two purposes [21]. Firstly, the gene of interest will be tagged endogenously with GFP, enabling the study of its expression by live imaging. Secondly, co-expression of FLP in a specific tissue or at a particular moment during development will excise the second exon of GFP, thereby introducing a reading frame shift and a premature termination codon that together will abolish expression of the gene of interest. Several of the GFP knock-in strains in Table 1 contains this GFP variant. (B) For FLP-controlled complete removal of the gene of interest, an upstream GFP cassette with one or two Frt sites can be combined with insertion of a Frt site in the 3' UTR of the gene of interest. (C) Inducible degradation of GFP-tagged protein using a *vhhGFP4::zif-1* transgene [27] downstream of a Frt-flanked mCherry::HIS-58 stop cassette. VHHGFP4::ZIF-1 triggers ubiquitination of GFP-tagged proteins, thus enabling rapid and specific degradation in a spatiotemporal controlled manner. (D) DamID experiments for mapping of protein-DNA contacts rely on low expression of Dam fusion proteins [14,29]. The FLP/Frt system allows tissue-specific basal expression of Dam fusion proteins from an uninduced heat shock promoter [24,25].

Table 1. *C. elegans* nucleoporins

C. elegans	Human	Mutant alleles[a]	FP knock-in[b]	Other strains[c]	Other reagents[d]	Reference
MEL-28	ELYS/ AHCTF1	*tm2434; t1578; t1684*	**BN426** (GFP)	BN208 (Dam)	Abs	[41,34,13]
NPP-1	NUP54	*syb207*		**LW1089** (GFP); **OCF22** (mCh);	Aff; Y2H	[42-44]
NPP-2	NUP85	*tm2199*	BN1044 (GFP)			Askjaer lab
NPP-3	NUP205	*ok1999*			Abs; Y2H	[45,43]
NPP-4	NUPL1	*ok617*		XA3548 (GFP)	Aff; Y2H	[44,43,46]
NPP-5	NUP107	*ok1966*; *tm3039*		**BN69** (GFP)	Abs	[47]
NPP-6	NUP160	*ok2821*; *tm4329*				
NPP-7	NUP153	*ok601*	WLP799 (GFP)	**JH2686** (GFP)	Abs	[48,49]; Pintard lab

NPP-8	NUP155	*tm2513*	WLP800 (mCh)	**XA3546**[e] (GFP)	Abs	[46]; Pintard lab
NPP-9	NUP358	*gk3059*	YY1566 (RFP)	**JH2184** (GFP)	Abs	[50,49,51]
NPP-10N[f]	NUP98	*ok467*		**JH2458** (GFP)	Abs	[48,49,47]
NPP-10C[f]	NUP96				Abs	[48,47]
NPP-11	NUP62	*ok1599; gk5507*		GFP	Aff; Y2H	[43,44,52]
NPP-12	NUP210	*ok2424*; *tm2320*	BN1136 (GFP), DG4460 (GFP)		Abs	[53,54]; Askjaer lab
NPP-13	NUP93	*ok1534*	WLP805 (GFP)		Abs; Y2H	[45,43]; Pintard lab
NPP-14	NUP214	*ok1389; sm160*		dsRed		[55]
NPP-15	NUP133	*ok1954*		BN75 (GFP)	Abs	[47,17]
NPP-16	NUP50	*ok1839*; *tm1596*			Abs	[12]
NPP-17/ RAE-1	RAE1	*ok1720*; *tm2784*; *tm2796*		mCh	Aff	[56]
NPP-18	SEH1	*ok3278*				
NPP-19	NUP35	*tm2886*	BN1015 (GFP)	**BN46** (GFP), BN510 (Dam)	Abs; Y2H	[47,57]; Askjaer lab
NPP-20	SEC13R			EGD496 (GFP)		[58]
NPP-21	TPR	*tm1541*; *tm2952*	BN1062 (GFP)			Askjaer lab
NPP-22 /NDC-1	NDC1/ TMEM48	*tm1845*	DG4557 (GFP), WLP801 (mCh)	**EU1485** (GFP), BN375 (Dam)	Abs	[59,60,54]; Pintard lab; Askjaer lab
NPP-23	NUP43			BN150 (GFP)		[47]
NPP-24	NUP88		BN739 (GFP)			Askjaer lab
NPP-25	TMEM33		BN1089 (GFP)			Askjaer lab
NPP-26	GLE1					
NPP-27	ZC3HC1					

No clear *C. elegans* homologues were found for the mammalian nups AAAS/ALADIN, NUP37, NUP188, NUPL2/hCG1, POM121.

[a] Only selected alleles are listed. Most of these and other alleles are available from the *Caenorhabditis* Genetics Center (CGC; University of Minnesota; https://cgc.umn.edu) and the National Bioresource Project for the Experimental Animal "Nematode *C. elegans*" (Tokyo Women's Medical University School of Medicine; http://www.shigen.nig.ac.jp/c.elegans/index.jsp).

[b-c] Strains expressing nucleoporin fusion proteins, either endogenously tagged[b] or as transgene[c]; Dam, DNA adenine methyltransferase; GFP, green fluorescent protein; mCh, mCherry; RFP, red fluorescent protein. Strain names are indicated when known; strains available from CGC are written in bold.

[d] Abs, antibodies; Aff, expression of affinity-tagged protein; Y2H, plasmids to study yeast two hybrid interactions.

[e] Prone to germ-line silencing.

[f] Because NPP-10N and NPP-10C are produced from a single protein precursor, a given RNAi phenotype will generally reflect the combined effect of depleting both proteins. P granule phenotypes are, however, specific to NPP-10N depletion.

Table 2. Strains stably expressing codon-optimized FLP

Promoter	Cell type	Strain	Reference[a]
ckb-3	Somatic gonad	BN854	unpublished
ceh-60	Multiple neurons and intestine	BN813	[61]
dat-1	Dopaminergic neurons	BN617	[21]
dpy-7	Hypodermal	BN551	[21]
eat-4	Glutamatergic neurons	BN993	unpublished
gpa-14	Neurons	BN816	unpublished
hlh-8	M lineage	BN502	[21]
hlh-12	Distal tip cell	BN1204	unpublished
hsp-16.41	Ubiquitous; heat inducible	BN646	[21]
lag-2	Multiple	BN558	[21]
lin-31	P lineage	BN1023	unpublished
mec-7	Mechanosensory neurons	BN498	[21]
mex-5	Germ line	BN711	[22]
myo-2	Pharyngeal muscle	BN543	[21]
myo-3	Body wall muscle	BN503	[21]
nhr-82	Seam cell lineage	BN455	[21]
nhx-2	Intestine	BN999	unpublished
rgef-1	Pan-neuronal	BN507	[21]
tph-1	Serotonin-producing neurons	BN499	[21]
UAS	Gal4-compatible	BN908	[23]
unc-17	Cholinergic neurons	BN1123	unpublished
unc-47	GABAergic motor neurons	BN544	[21]
unc-122	Coelomocytes	BN1029	unpublished

[a] Unpublished strains can be requested from the corresponding author.

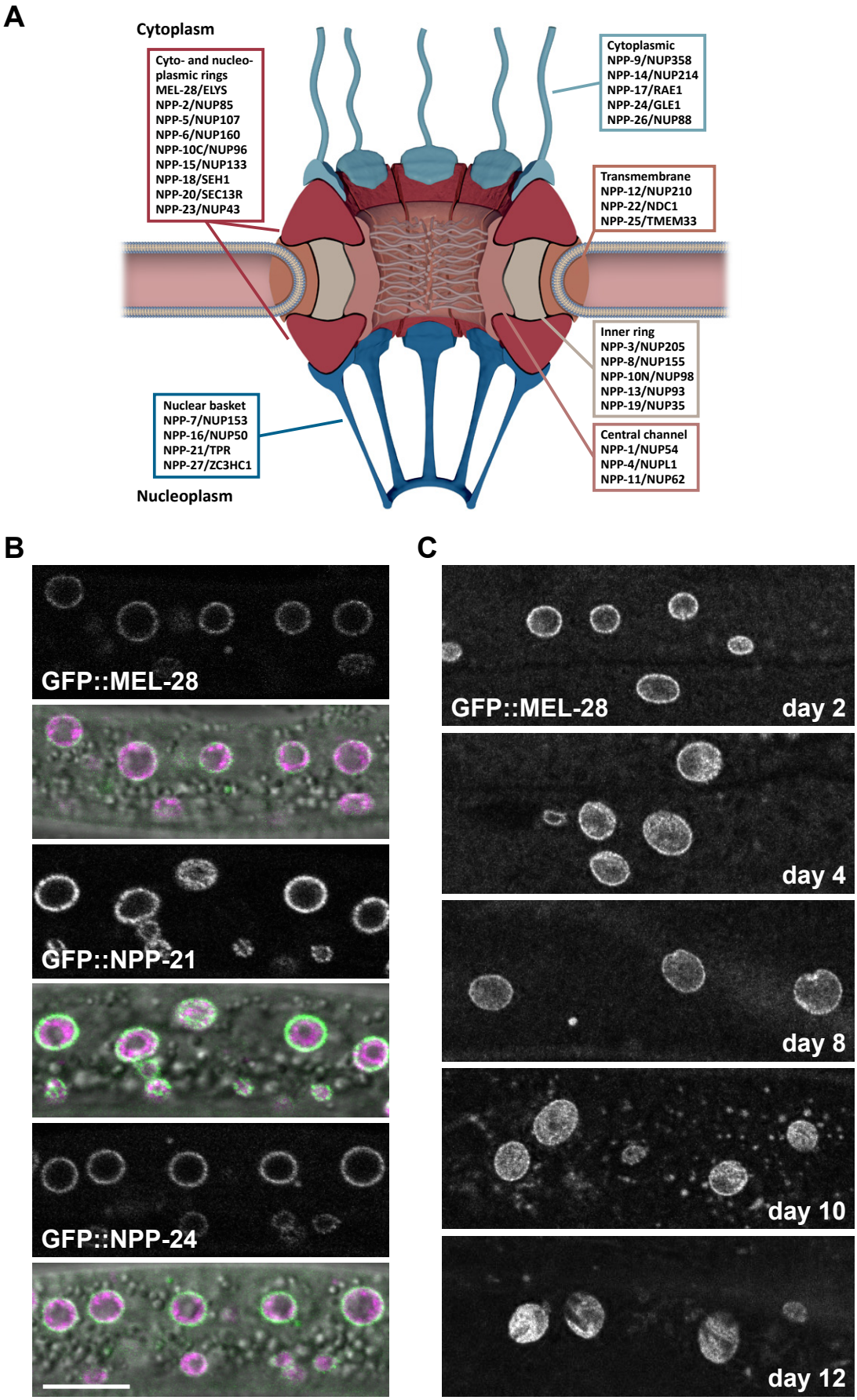
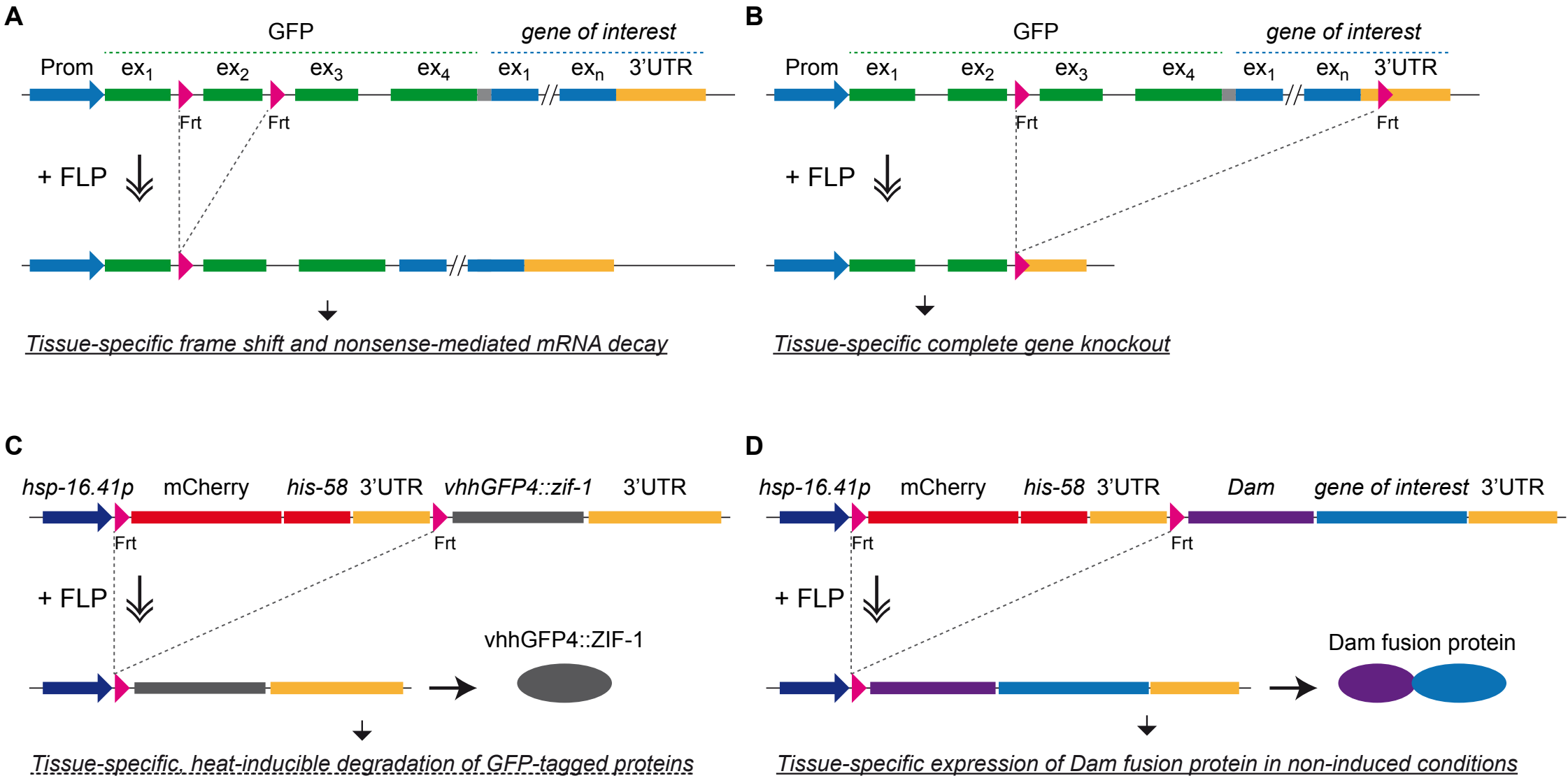

A
Cytoplasm
Cyto- and nucleo-plasmic rings
MEL-28/ELYS
NPP-2/NUP85
NPP-5/NUP107
NPP-6/NUP160
NPP-10C/NUP96
NPP-15/NUP133
NPP-18/SEH1
NPP-20/SEC13R
NPP-23/NUP43
Cytoplasmic
NPP-9/NUP358
NPP-14/NUP214
NPP-17/RAE1
NPP-24/GLE1
NPP-26/NUP88
Transmembrane
NPP-12/NUP210
NPP-22/NDC1
NPP-25/TMEM33
Inner ring
NPP-3/NUP205
NPP-8/NUP155
NPP-10N/NUP98
NPP-13/NUP93
NPP-19/NUP35
Central channel
NPP-1/NUP54
NPP-4/NUPL1
NPP-11/NUP62
Nuclear basket
NPP-7/NUP153
NPP-16/NUP50
NPP-21/TPR
NPP-27/ZC3HC1
Nucleoplasm
B
GFP::MEL-28
GFP::NPP-21
GFP::NPP-24
C
GFP::MEL-28
day 2
day 4
day 8
day 10
day 12
D
GFP::NPP-21

Figure 2


A
GFP
gene of interest
Prom
ex1
ex2
ex3
ex4
ex1
exn
3'UTR
Frt
Frt
+ FLP
Tissue-specific frame shift and nonsense-mediated mRNA decay
B
GFP
gene of interest
Prom
ex1
ex2
ex3
ex4
ex1
exn
3'UTR
Frt
Frt
+ FLP
Tissue-specific complete gene knockout
C
hsp-16.41p
mCherry
his-58
3'UTR
vhhGFP4::zif-1
3'UTR
Frt
Frt
+ FLP
vhhGFP4::ZIF-1
Tissue-specific, heat-inducible degradation of GFP-tagged proteins
D
hsp-16.41p
mCherry
his-58
3'UTR
Dam
gene of interest
3'UTR
Frt
Frt
+ FLP
Dam fusion protein
Tissue-specific expression of Dam fusion protein in non-induced conditions