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Supplementary Information for

Modeling the Transport of Nuclear Proteins Along Single Skeletal Muscle Cells

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Supplementary Materials and Methods

Plasmid Design

DNA sequences for the RFP-cNLS constructs were designed based on sequences for monomeric (mCherry, GenBank: AY678264.1), tandem dimeric (tdTomato, GenBank: AY678269.1) and tetrameric (DsRed-Express2, GenBank: FJ226077.1) variants of the RFP protein isolated from *Discosoma sp*. The original stop codons were removed and three copies of the SV40 cNLS (DPKKKRKV) plus a new stop codon were added to the 3' ends of these sequences. The sequences were synthesized (BioCat) and inserted into the pTetOne Inducible Expression Vector (Clontech) for transient transfection and pFUW-TetO-MCS (Addgene #84008) for lentiviral production. Plasmid FUW-M2rtTA (Addgene #20342) encoding the lentiviral tetracycline transactivator was purchased. Plasmid pTetOne-Six1-myc was generated by synthesizing the protein coding sequence of mouse Six1 (GenBank: Q62231) with three myc tags (EQKLISEEDL) added before the stop codon (BioCat). The sequence was inserted into pTetOne (Clontech). Construction of pARNT-CFP was described previously (1). The expression vector used to express red and green fluorescent proteins targeted to mitochondria contained either DsRed2 or AcGFP1 fused with a mitochondrial localization sequence (BD Pharmingen #558722 and 555718). The expression vector used to express green fluorescent protein was pmax-GFP (Amaxa). In both inducible and constitutive constructs, all promoters are derived from the cytomegalovirus (CMV) promoter.

Myoblast Transfection

A solution containing a 45:1 polymer:DNA mass ratio was prepared in complexation buffer (20 mM HEPES (Sigma #H3375), 50 mg/mL glucose (Sigma #G8270) in water, pH 7.4). After mixing, the solution was immediately vortexed and 10 µL (150 ng plasmid DNA) were used in each transfection. After four hours, the transfection solution was replaced with differentiation medium (5% horse serum (ThermoFisher #26050070) and 1% (v/v) penicillin-streptomycin in DMEM). After 2 days, the myoblasts had fused into multinucleated myotubes.

Lentivirus Production

HEK293FT cells (Invitrogen) were seeded in 75 cm^2 flasks, cultured in DMEM (Invitrogen) containing 10% FBS (Gibco) and 25 µg/mL gentamicin (Gibco), and were transfected by Lipofectamine 2000 (Invitrogen) with the following plasmids: 5.1 µg of pMD2.G (Addgene plasmid 12259), 9.7 µg of psPAX2 (Addgene plasmid 12260), and 15.0 µg of pFUW-TetO-RFP or FUW-M2rtTA for four hours in OptiMEM medium (Gibco). The medium was changed following transfection and again after 14 hours. Seventy-two hours after transfection, the medium was collected in 50 ml tubes and filtered through a 0.45 µm filter to remove cell and membrane debris. The supernatant was then concentrated to 100× in Amicon Ultra centrifugal filter tubes (Millipore), and the concentrated virus aliquoted and stored at −80 °C.

Lentivirus Transduction and Cell Sorting

Myoblasts were seeded in collagen-coated 6-well plates and cultured in growth medium. In each well, cells were transduced with 10 µL of RFP lentivirus and 5 µL of transactivator lentivirus. Cells were expanded over five days in the presence of 2 µg/mL doxycycline to induce RFP expression. RFP^+ myoblasts were sorted by FACS Jazz (BD) and collected into 10 cm^2 dishes. Cells were expanded without doxycycline leading to loss of RFP expression, frozen, and stored in the liquid nitrogen vapor phase.

EdU Labeling

Previously-sorted RFP^+ myoblasts were seeded in collagen-coated 25 cm^2 culture flasks and cultured in growth media containing 10 µM 5-ethynyl-2'-deoxyuridine (EdU) for 24 hours. Cells were then collected and seeded at a density of 50,000 cells/well into 96-well plates coated with 0.1 mg/mL poly-L-lysine hydrobromide (Sigma #P6282) and a solution of 50 µg/mL type I collagen, 10 µg/mL fibronectin (Sigma # F1141) and 10 µg/mL laminin (Sigma #L2020) in phosphate buffered

saline (ThermoFisher #14190) at a ratio of 1:50 with wildtype myoblasts. After one hour, growth media was changed to differentiation media to induce myoblast fusion. After 48 hours, fresh media containing 2 µg/mL doxycycline was added to induce RFP expression. After 72 hours, cells were fixed and labeled with Hoechst 33342 (Sigma #B2261) and the Click-iT™ Plus EdU Alexa Fluor™ 488 Kit (Invitrogen #C10637) according to the manufacturer's protocol.

In Situ Hybridization

In situ hybridization was performed on myotubes derived from fusion of myoblasts, as described in Material and Methods "Cell culture" section, on 4 chamber culture slides (Millicell EZ slides, #354114) coated with 0.1 mg/mL poly-L-lysine hydrobromide (Sigma #P6282), 50 µg/mL type I collagen, 10 µg/mL fibronectin (Sigma # F1141) and 10 µg/mL laminin (Sigma #L2020). Briefly, cells were fixed in 4% paraformaldehyde (PFA, Sigma #P6148) in phosphate buffer (PBS), washed three times with PBS, incubated with Proteinase K solution (1 µg/ml Proteinase K in 50 mM TRIS.CL pH 7.5, 5 mM EDTA) for 5 min and PFA-fixed and washed with PBS as before. Slides were subsequently treated with an acetylating solution (2 mM HCl water solution containing 14 µl/ml triethanolamide, 2.5 µl/ml acetic anhydride) for 10 minutes, washed with PBS and incubated with hybridization solution (50% formamide, 5x saline-sodium citrate (SSC) buffer, 5x Denhardt's Solution, 250 µg/ml Yeast RNA, 500 µg/ml Herring sperm DNA, 20 mg/ml blocking reagent from Roche) for 1 hour in a chamber humidified with a 50% formamide/5X SSC solution. All steps were performed at room temperature (RT). Slides were subsequently incubated overnight at 70°C with labelled probe diluted in hybridization solution, followed by washes with a 0.2X SSC solution at 70°C for 1 hour and with B1 solution (0.1 M Tris-Cl pH 7.5, 0.15 M NaCl, 10% heat inactivated fetal bovine serum) for 1 hour at RT, and an overnight incubation with anti-digoxigenin-alkaline phosphatase Fab fragments antibody (Roche #11093274910) diluted in B1 solution at 4°C in a water humidified chamber. Slides were then washed with B1 solution and equilibrated in B3 solution (0.1M Tris-Cl pH 9.5, 0.1M NaCl, 50mM $MgCl_2$) before incubation with developing solution (10% polyvinyl alcohol, 100 mM Tris-Cl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.24 mg/ml levamisol) containing NBT (Roche #11383213001) and BCIP (Roche #11383221001) at RT. The developing reaction was stopped by placing the slides in water, and after several washes the slides were mounted using Aquatex mounting medium (Merck #1085620050). Labelled probes were produced from *in vitro* transcription of linearized plasmid containing the sequence for mCherry, tdTomato or DsRed-Express2 using the Dioxigenin RNA labeling kit (Roche #11175025910) according to the manufacturer's protocol. Probes were purified using Microspin G-50 columns (GE Healthcare #W9838404) and stored at -20 °C.

Western Blots and RFP Half-life Quantification

Sorted RFP^+ myoblasts were seeded at 1.5 million cells/well in collagen coated 6-well plates in growth media. After four hours to allow for cell attachment, media was changed to differentiation media. After 48 hours, differentiation media was replenished with 2 µg/mL doxycycline to induce RFP expression. Negative controls (NC) were not given doxycycline. After 72 hours, cells were washed once with PBS and given fresh differentiation media without doxycycline. Protein was collected at subsequent timepoints using doxycycline withdrawal as T=0. Cell extracts were lysed in RIPA buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% (w/v) TritonX-100; 0.5% (w/v) Na-deoxycholate; 0.1% (w/v) sodium dodecyl sulfate (SDS); 20 mM glycerol-2-phosphate; 5 mM sodium pyrophosphate) supplemented with phosphatase and protease inhibitors and total protein was extracted. Protein concentration was determined by Bradford Protein Assay following manufacturer's instructions (BioRad). 10 µg of protein were separated by SDS polyacrylamide gel membranes (PAGE) and transferred to PVDF membranes. After blocking with 5% skim milk, blots were incubated overnight at 4 ºC with Anti-RFP primary antibody cocktail (Rockland #600-401-379) diluted in 0.1% bovine serum albumin (BSA). After washing, membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for one hour at room temperature. Blots were visualized by enhanced chemiluminescence (GE Healthcare) and exposed on Chemidoc (BioRad). Band densitometry was performed using FIJI (Fiji Is Just ImageJ, http://fiji.sc) and half-lives were determined by fitting a first-order exponential decay to the intensity values (Prism 8, GraphPad).

Reporter Protein Imaging and Analysis

Time-lapse epifluorescence microscopy was used to monitor transport of the RFP-cNLS fusion proteins in living myotubes. Cultures of transfected myotubes were stained with 100 ng/mL Hoechst 33342 (Sigma #B2261) for 30 minutes at 37 °C in differentiation medium. Afterwards the medium was replaced with fresh differentiation medium supplemented with 2 µg/mL doxycycline to induce RFP-cNLS expression. The production and transport of the RFP-cNLS proteins were then monitored for 24 hours (20-minute imaging interval) with temperature and carbon dioxide control (Zeiss Cell Observer).

For bulk analyses, myoblasts were fixed with 3.7% formaldehyde for 20 minutes at room temperature and stained with 10 µg/mL Hoechst for 10 minutes. For analyses requiring visualization of mitochondria, 500 nM of MitoTracker Deep Red (Invitrogen #M22426) was added to culture media for 30 minutes prior to fixation. Whole well scan slides were used to image the RFP-cNLS fusion proteins and nuclei within differentiated myotubes (Olympus IX73). ImageJ was used to quantify the relative distribution of RFP-cNLS within myonuclei. Briefly, myonuclei within transfected myotubes with greater than 5 nuclei were outlined and the background-corrected average RFP fluorescence and position of each nucleus were measured. The nucleus with the highest average RFP fluorescence within a myotube was assumed to be the transfected myonucleus and all other nuclei were considered 'neighboring'. The average fluorescence of all 'neighboring' myonuclei were normalized to the transfected nucleus and their linear distances from the transfected nucleus were calculated. The 'neighboring' nuclei were binned (bin size = 50 µm) based on their distances from the transfected nucleus and the relative RFP signal intensities were plotted versus distance from the transfected nucleus. The quantitative results presented in Fig. 2B are used as controls for comparison in Fig. 3. The results presented in Fig. 4 are used as controls in Fig. S6. To measure myotube width, ImageJ was used to measure the widest section of Hoechst-stained myotubes.

To quantify the distribution of the fluorescent proteins fused with a mitochondrial localization signal, the average intensity of the fluorescent construct within 10 µm sections of the myotubes were normalized to the average MitoTracker fluorescent intensity within that same section. Distances and fluorescent intensities were then normalized to the section of the myotube with the highest local signal. Data were binned by distance (bin size = 50 µm) and were plotted versus distance from the section of the myotube with the highest local signal.

Immunocytochemistry

For targets requiring immunocytochemical detection (Six1-myc and fibrillarin), cells were fixed with 3.7% formaldehyde in DPBS with Ca/Mg^{++} for 20 minutes at room temperature, permeabilized with 0.2% Triton X-100 for five minutes, and blocked with 1% bovine serum albumin for 30 minutes. Primary antibodies against fibrillarin (mouse monoclonal, Abcam # ab4566) or c-Myc (rabbit polyclonal, Sigma # C3956) were added at 1:500 dilution for 2 hours followed by washing and incubation with secondary antibodies (Goat anti-Mouse IgG AF488 (Invitrogen# A-11001) or Goat anti-Rabbit IgG AF488 (Invitrogen# A-11008)) at 1:1000 for 1 hour. Nuclei were counterstained with DAPI (Life Technologies# D1306) at 500 ng/mL for five minutes prior to imaging. Cells were imaged on the Cell Observer widefield (Zeiss) or LSM700 confocal (Zeiss) microscope.

Mathematical Model

To model RFP-cNLS transport, MATLAB (MathWorks) was used to simulate a symmetrical cylindrical myotube with spherical myonuclei evenly distributed along its center. The 'transfected' myonucleus was positioned at the center of the myotube (left boundary of the model). The production of RFP-cNLS was assumed to be constant with time and to decrease linearly over 150 µm from this boundary. A reflecting boundary ($\nabla C = 0$) was imposed at the end of the myotube (right boundary) to prevent proteins from exiting the simulation. The transport of the RFP-cNLS was modeled only in the longitudinal-dimension (radial and azimuthal symmetry were assumed). The simulation included cytoplasmic diffusion, protein half-life, facilitated nuclear import, and diffusion across the NPC, leading to the governing equation:

$$\frac{dC}{dt} = D\nabla^2 C - kC - IC + D_N(C_N - C),$$

where *D* is the cytoplasmic diffusion coefficient, *k* is the protein half-life, *I* is the facilitated nuclear import function, D_N is a coefficient governing the rate of diffusion across the NPC, and C_N is the local nuclear concentration of RFP-cNLS. The facilitated import function, *I,* was a step function that was defined as a positive constant in the perinuclear areas of the myotube and as zero elsewhere. To approximate these coefficients, we relied on published diffusion coefficients for proteins in cytoplasm,[28] measurements of nuclear import rates,[23] NPC density[29] and fluorescent protein half-life[30]. Morphological parameters (i.e. nucleus diameter, myotube width, etc.) were approximated based on observations of myotubes in culture, leading to an initial set of parameters for the simulation (Table 1).

Statistical Analysis

Statistical analyses were performed using Prism 8 (GraphPad Software, Inc.). Differences between more than two groups were assessed by two-way analysis of variance with a Bonferroni post-test to compare means. An unpaired t-test was used to compare means between two groups. A p-value less than 0.05 was considered statistically significant. All data are presented as mean ± standard error of the mean (SEM).

Supplementary Movie Legends

Supplementary Movie 1 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible DsRed-cNLS fusion protein. Movie shows fluorescent signal from the DsRed fusion protein. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

Supplementary Movie 2 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible DsRed-cNLS fusion protein. Movie shows fluorescent signal from the Hoechst-stained myonuclei. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

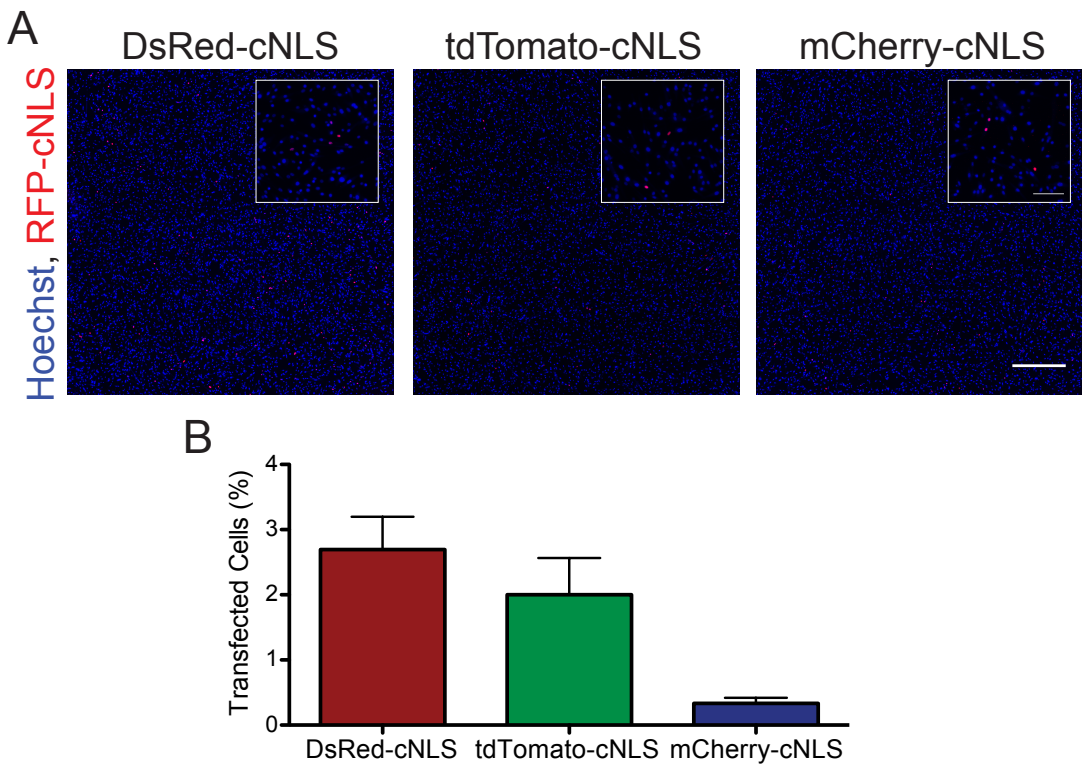
Supplementary Movie 3 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible tdTomato-cNLS fusion protein. Movie shows fluorescent signal from the tdTomato fusion protein. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

Supplementary Movie 4 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible tdTomato-cNLS fusion protein. Movie shows fluorescent signal from the Hoechst-stained myonuclei. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

Supplementary Movie 5 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible mCherry-cNLS fusion protein. Movie shows fluorescent signal from the mCherry fusion protein. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

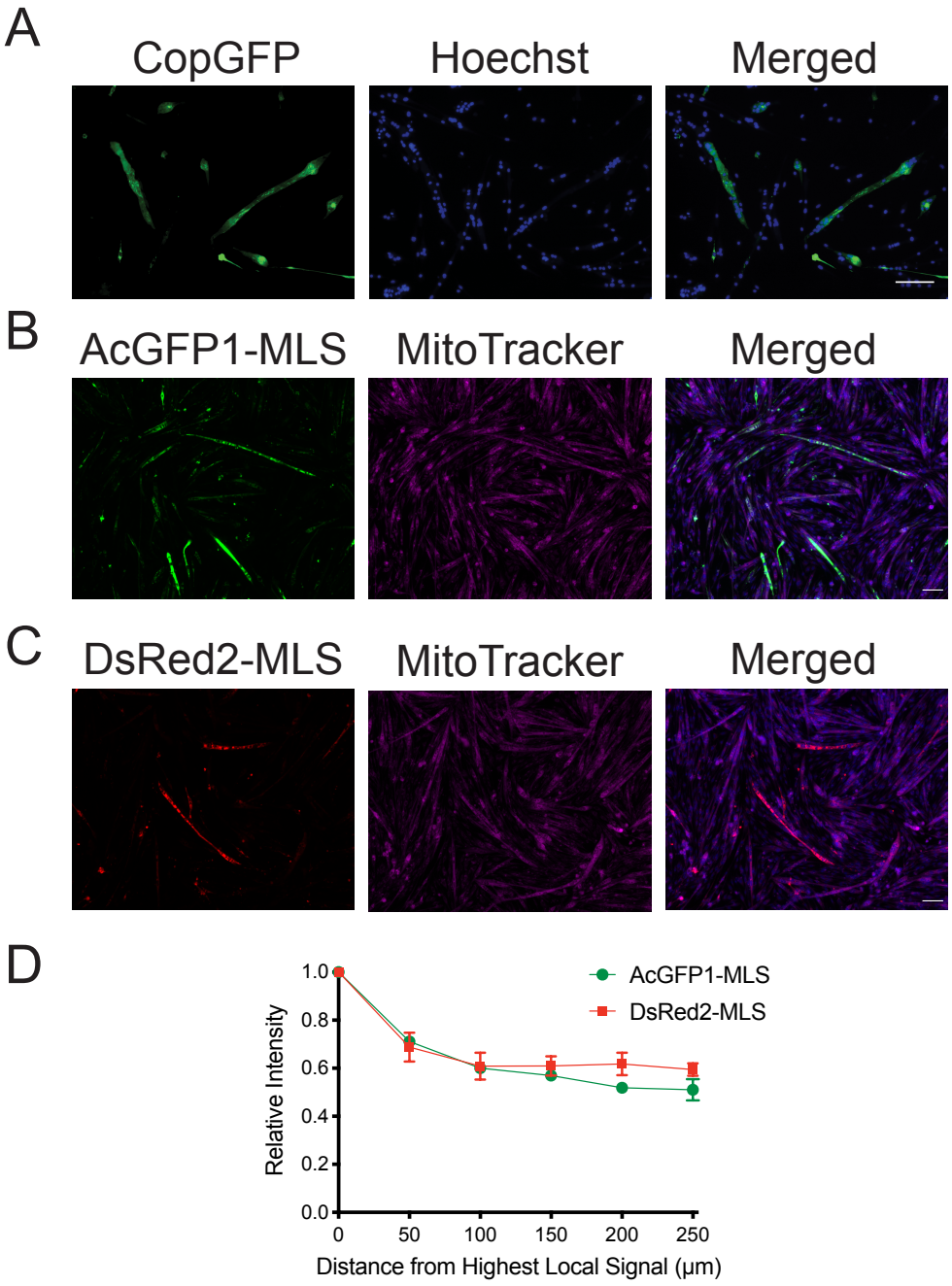
Supplementary Movie 6 (separate file): Time-lapse epifluorescence microscopy of a Hoechst-stained myotube that expresses the doxycycline-inducible mCherry-cNLS fusion protein. Movie shows fluorescent signal from the Hoechst-stained myonuclei. Time stamp is in hours and indicates time since addition of doxycycline. The scale bar represents 20 μm.

Figure S1



Supplementary Figure 1: Transfection efficiency of tetracycline-inducible RFP-cNLS expression vectors. Primary mouse myoblasts were transfected with tetracycline-inducible DsRed-cNLS, tdTomato-cNLS, or mCherry-cNLS expression vectors one hour after seeding. After four hours of transfection, the culture medium was replaced with myoblast differentiation medium containing 2 µg/mL doxycycline. A) After one day, a small fraction of myoblasts expressed the RFP-cNLS fusion proteins, which had become localized to the myonuclei (insets). B) The percentage of transfected myoblasts was determined by quantifying the RFP-fluorescence intensity of myonuclei within a large culture area. A myoblast was considered positive if its myonuclear fluorescence intensity was greater than two-times the median myonuclear fluorescence intensity within the culture area. Data is presented as mean ± SEM. The scale bar represents 500 µm in the large images and 100 µm in the insets.

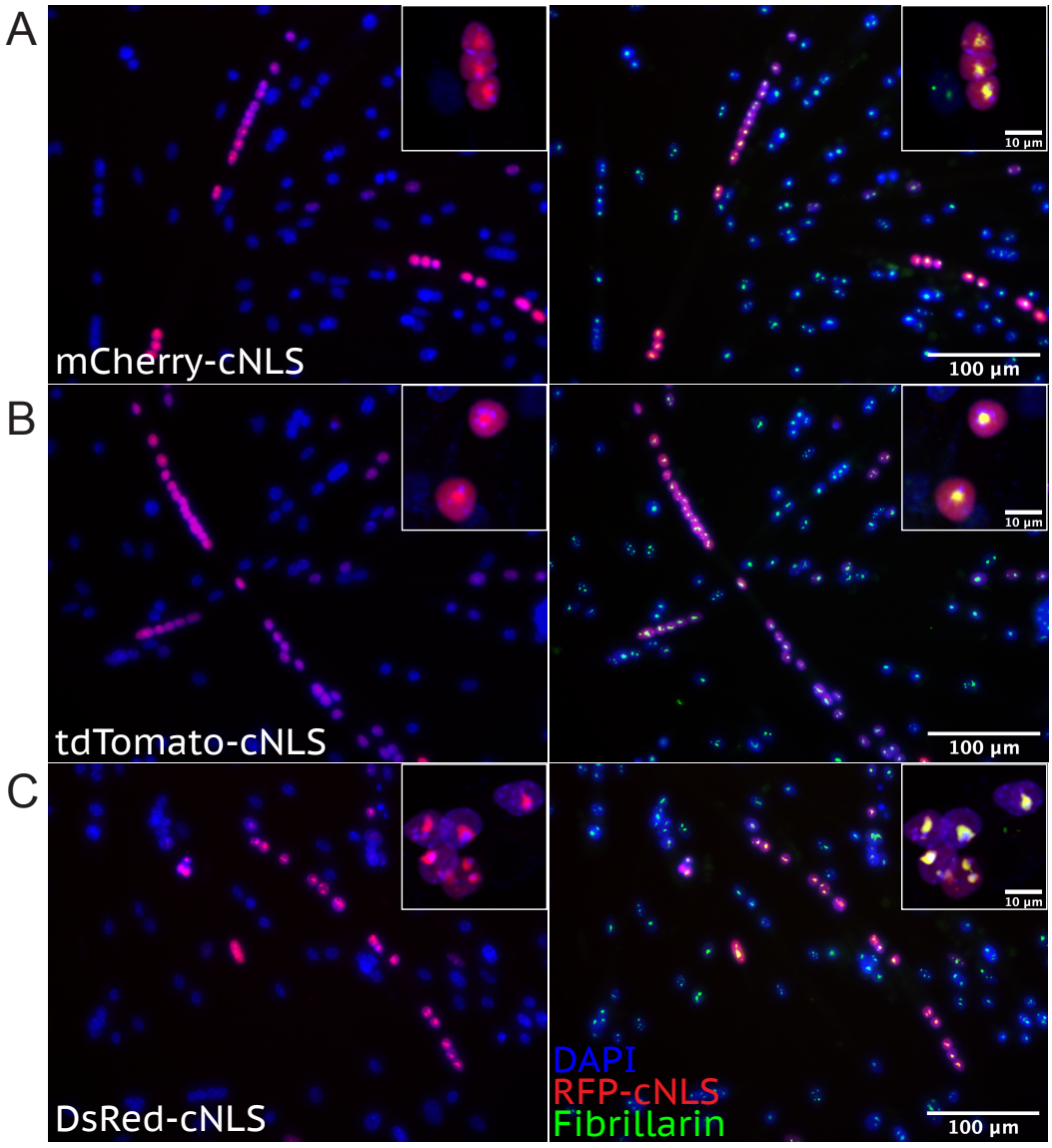
Figure S2



Supplementary Figure 2: Distribution of AcGFP1 and DsRed2 fused with a mitochondrial localization sequence within myotubes. Primary mouse myoblasts were sparsely transfected with expression vectors for A) an untargeted green fluorescent protein (CopGFP), B) AcGFP1 fused with a mitochondrial localization sequence (AcGFP1-MLS), or C) DsRed2 fused with a mitochondrial localization sequence (DsRed2-MLS). After myoblast fusion, fluorescent reporters

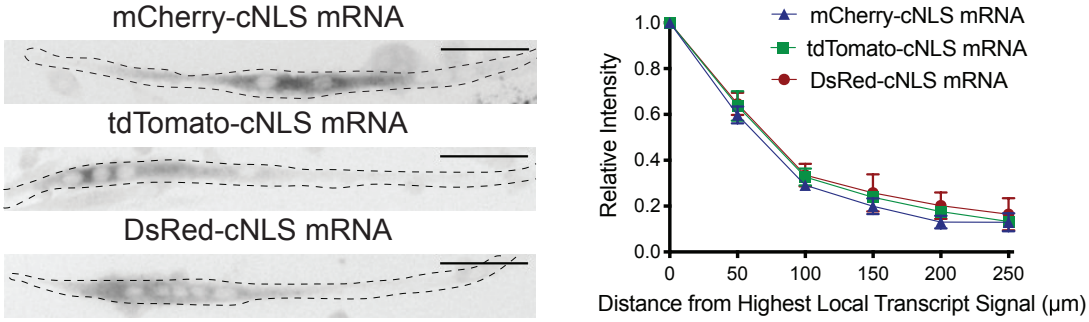
were distributed throughout the fused myotubes. MitoTracker dye was used to visualize all mitochondria. D) Despite differences in molecular weight between AcGFP1-MLS (26.9 kDa) and DsRed2-MLS (103.2 kDa), there were no signifcant differences in the weak gradients formed by these fusion proteins upon quantification. The scale bars represent 100 μm.

Figure S3



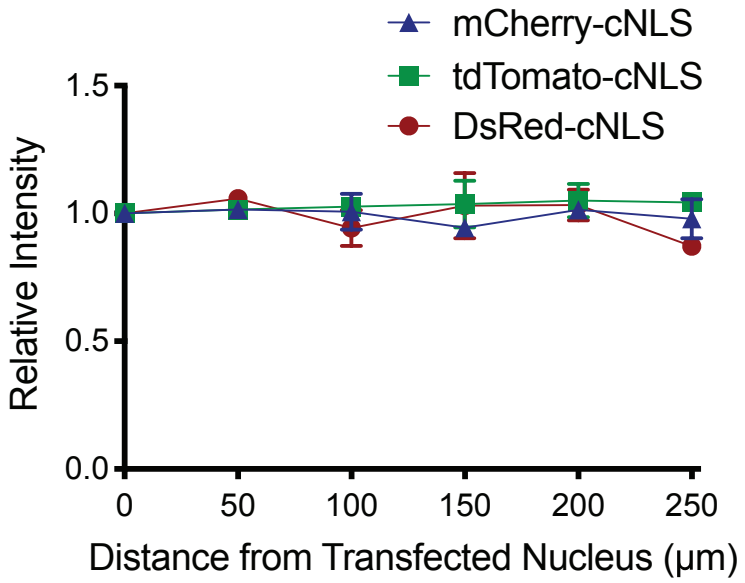
Supplementary Figure 3: RFP-cNLS proteins partially co-localize with the nucleolus. Primary mouse myoblasts were sparsely transfected with inducible expression vectors for RFP-cNLS fusion proteins of varying molecular weight (mCherry, tdTomato, or DsRed) and then differentiated for two days. After 24 hours of doxycycline induction, the myotubes were fixed and treated for immunocytochemical analysis. Labeling with anti-fibrillarin antibodies revealed partial subnuclear co-localization with (A) mCherry-cNLS, (B) DsRed-cNLS, and (C) tdTomato-cNLS.

Figure S4



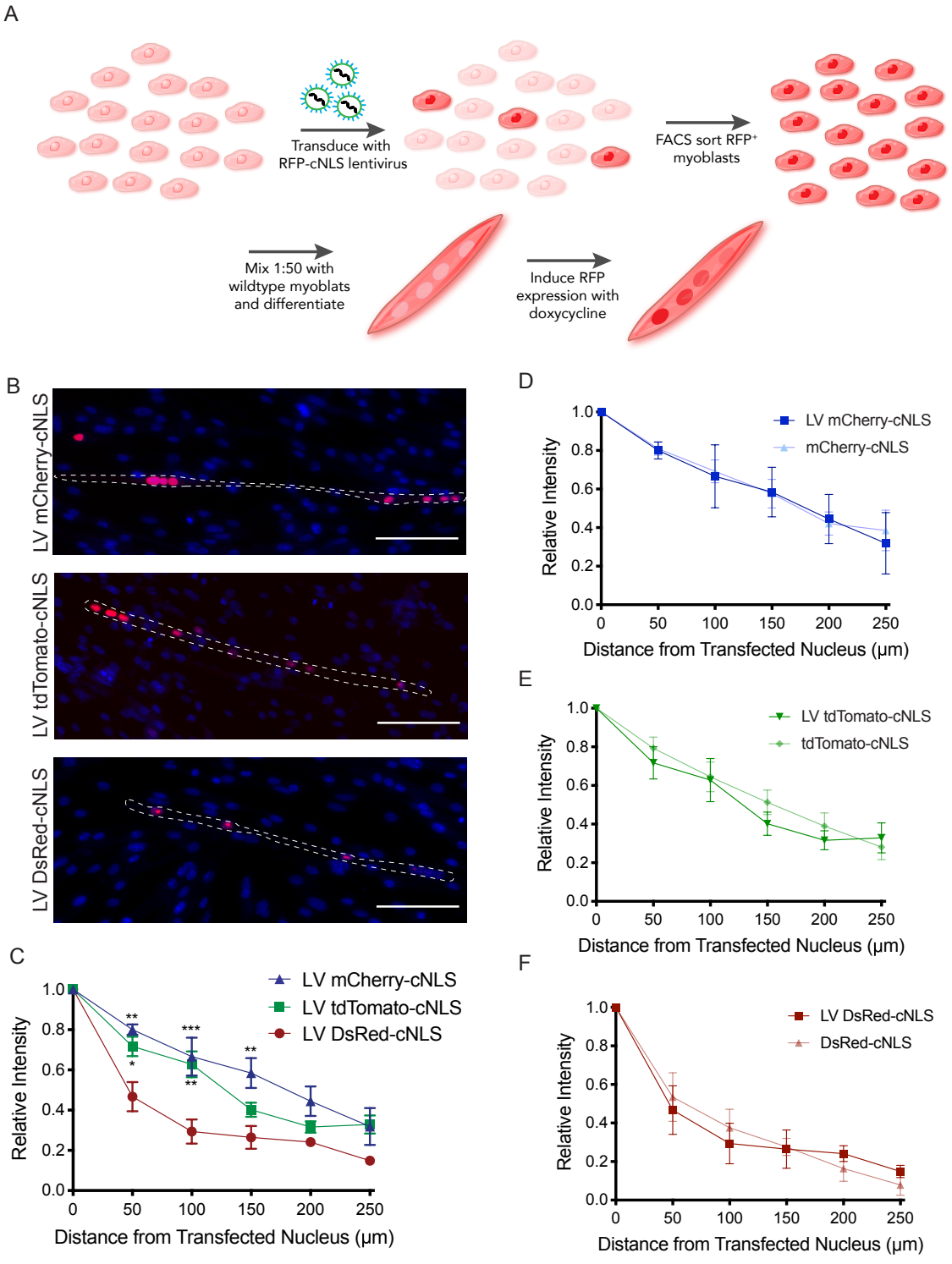
Supplementary Figure 4: Distribution of RFP-cNLS transcripts within myotubes. Primary mouse myoblasts were sparsely transfected with inducible expression vectors for RFP-cNLS fusion proteins of varying molecular weight (mCherry, tdTomato, or DsRed) and then differentiated for two days. After 24 hours of doxycycline induction, the distributions of transcripts for the three constructs were visualized by *in situ* hybridization (left). Transcript distribution was quantified by comparing the relative intensity of the *in situ* hybridization signal. Distances and intensities were normalized to the section of the myotube with the highest local transcript. Data are binned by distance (bin size = 50 μm) and are represented as mean ± SEM. The dotted lines show the approximate boundaries of the transfected myotubes. The scale bars represent 50 μm.

Figure S5



Supplementary Figure 5: Intensity of Hoechst nuclear staining is uniform along myotube length. Primary mouse myoblasts were sparsely transfected with inducible expression vectors for RFP-cNLS fusion proteins of varying molecular weight (mCherry, tdTomato, or DsRed) and then differentiated for two days. After 24 hours of doxycycline induction, the myotubes were fixed and the fluorescence intensity of Hoechst-stained myonuclei was quantified by measuring the positions and average intensity of myonuclei within individual transfected myotubes. Distances and fluorescence intensities were normalized to the brightest RFP myonucleus within each myotube. Data are binned by distance (bin size = 50 μm) and are represented as mean ± SEM. No significant differences were detected in Hoechst staining intensity across RFP isoforms or along myotube length.

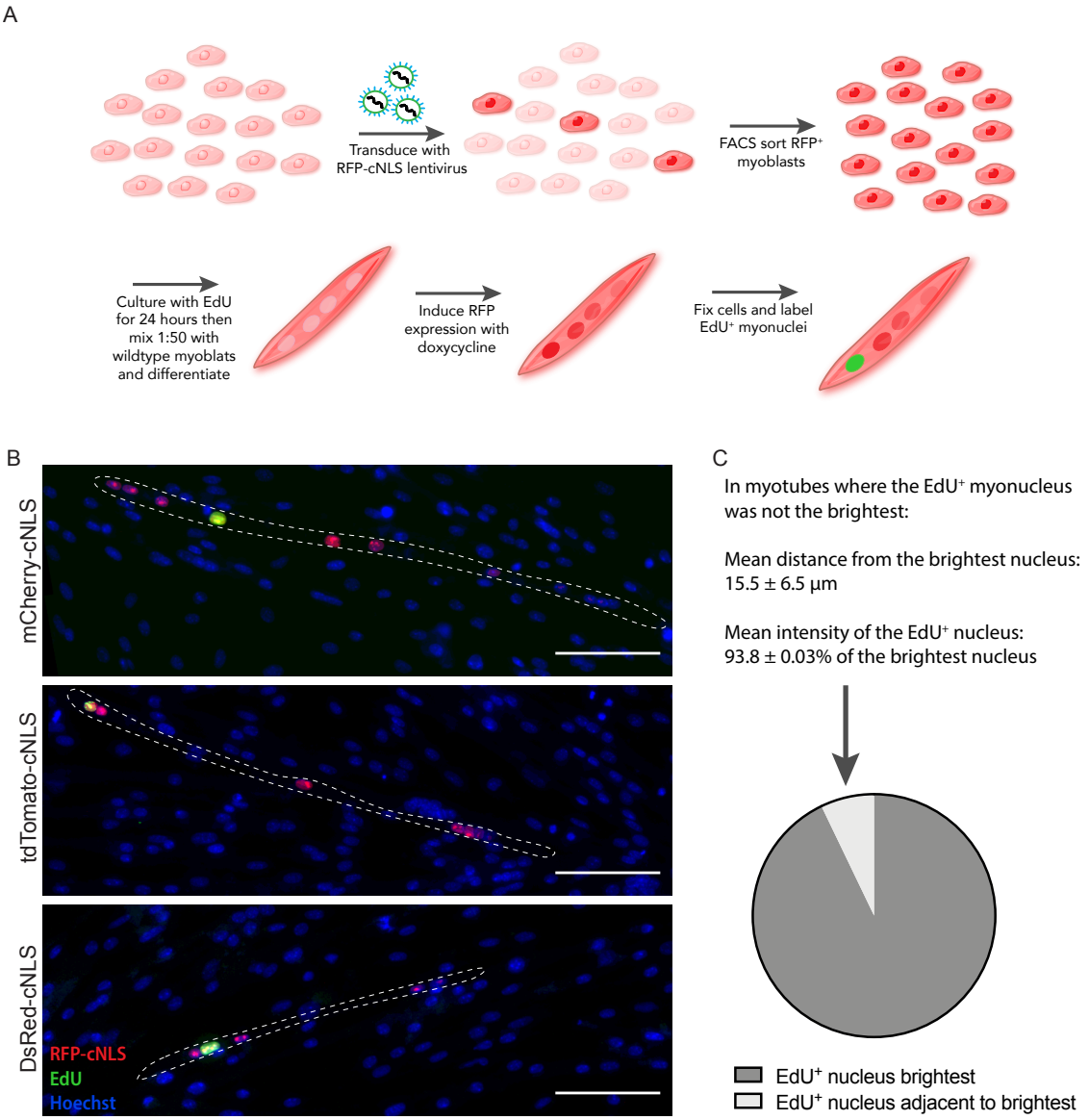
Figure S6



Supplementary Figure 6: Myonuclear propagation of RFP-cNLS proteins expressed by exogenous or integrated constructs is similar. A) Myoblasts were lentivirally-transduced with

inducible expression vectors for RFP-cNLS fusion proteins of varying molecular weight (mCherry is ~28.8 kDa; tdTomato is ~55kDa; DsRed forms a tetramer that is ~110kDa) along with the required tetracycline-induced transactivator cassette. RFP expression was induced to enable fluorescence-activated cell sorting of RFP-expressing cells. Following FACS, doxycycline withdrawal eliminated RFP expression. B) Sorted myoblasts bearing an RFP-cNLS cassette were mixed 1:50 with wildtype myoblasts and differentiated for two days. Following a subsequent 24-hour induction with doxycycline, the distributions of RFP fluorescence within Hoechst-stained myotubes were visualized by epifluorescence microscopy. C) RFP fluorescence was quantified by measuring the positions and average intensity of myonuclei within individual transfected myotubes. Distances and fluorescence intensities were normalized to the brightest myonucleus within each myotube (assumed to be the transfected nucleus). Data are binned by distance (bin size = 50 μm) and are represented as mean ± SEM (two-way ANOVA with Bonferroni posttest). D-F) Propagation behavior of RFP-cNLS proteins derived from lentiviral integrative expression closely matched the propagation previously observed with exogenous gene expression. The scale bars represent 100 μm in B. (*) $p<0.05$, (**) $p<.01$, (***) $p<.001$.

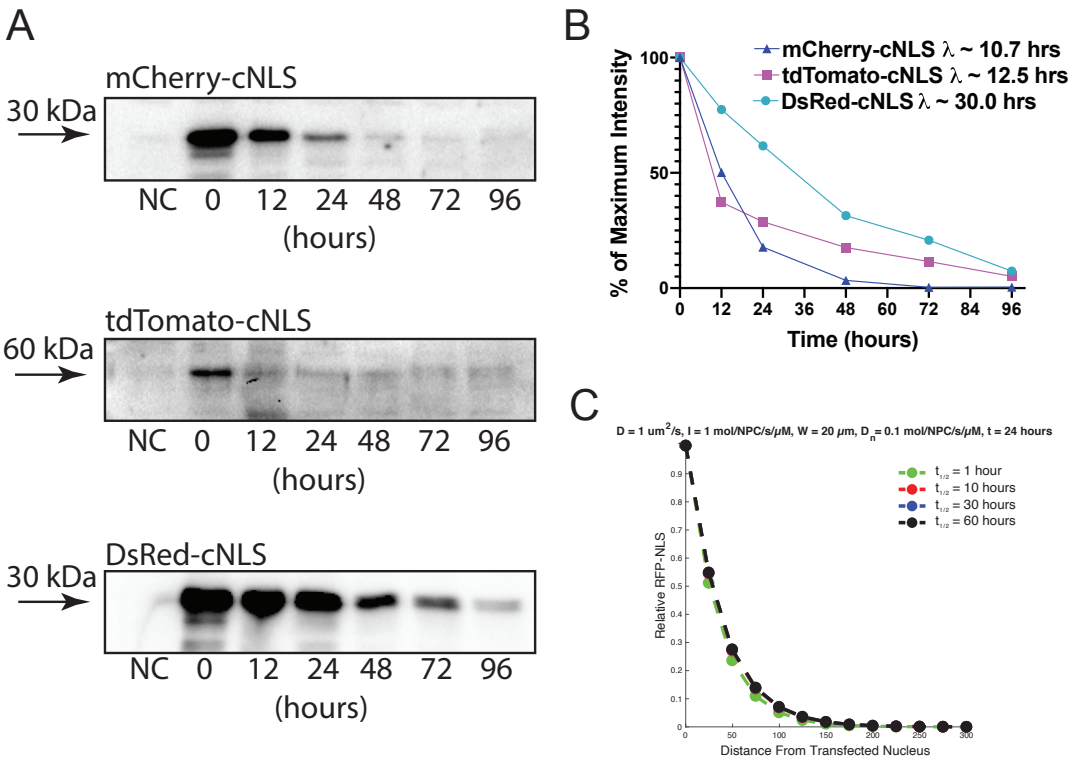
Figure S7



Supplementary Figure 7: Population tracking of myonuclei identifies the location of RFP-cNLS expression cassette. A) Myoblasts were lentivirally-transduced with inducible expression vectors for RFP-cNLS fusion proteins along with the required tetracycline-induced transactivator cassette. RFP expression was induced to enable fluorescence-activated cell sorting of RFP-expressing cells. Following FACS, doxycycline withdrawal eliminated RFP expression. Prior to use, RFP$^+$ myoblasts were cultured in the presence of EdU for 24 hours to enable their subsequent identification within differentiated myotubes. B) EdU-labeled, sorted myoblasts bearing an RFP-cNLS cassette were mixed 1:50 with wildtype myoblasts and differentiated for two days. Following a subsequent 24-hour induction with doxycycline, the cells were fixed and probed for EdU detection. Distributions of RFP fluorescence within Hoechst-stained myotubes, along with EdU functionalized with AF488,

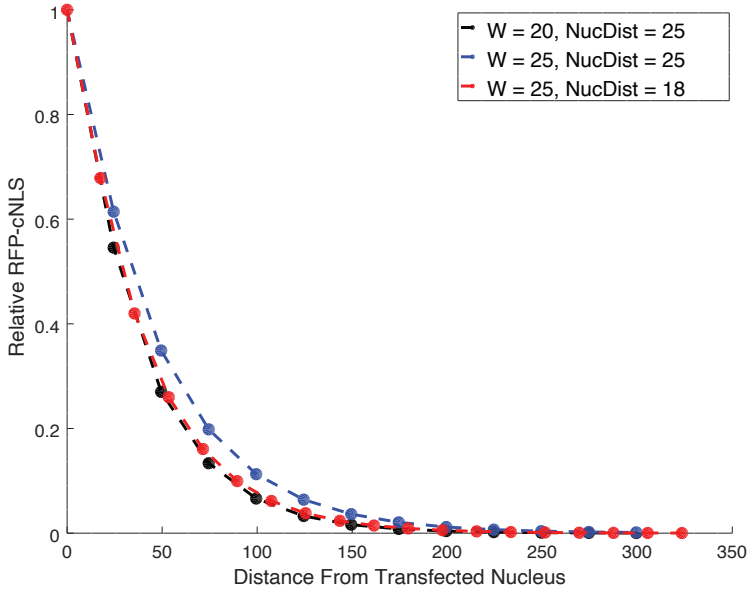
were visualized by epifluorescence microscopy. C) RFP fluorescence was quantified by measuring the positions and average intensity of myonuclei within individual transfected myotubes. Distances and fluorescence intensities were normalized to the brightest myonucleus within each myotube. In 93% of myotubes, the brightest nucleus corresponded to the EdU^+ nucleus. In the remaining myotubes, the EdU^+ nucleus was found 15.5 ± 6.5 μm from the brightest nucleus and possessed a mean intensity of 93.8 ± 0.03 % of the brightest nucleus, on average. There were no instances where the EdU^+ nucleus, and thus the RFP-cNLS expression cassette, were found separated from the 'peak' of the propagation gradient. The scale bars represent 100 μm in B.

Figure S8



Supplementary Figure 8: Half-lives of RFP-cNLS proteins in differentiated myotubes. Induced expression of RFP-cNLS in differentiated myotubes was stopped by removal of doxycycline. A) Western blots were used to detect the amount of RFP-cNLS protein remaining at various times following doxycycline withdrawal. B) Gel bands were quantified with band densitometry, and the values were used to fit first-order exponential decay curves to determine protein half-life (λ) in hours. The tetrameric DsRed-cNLS persists longer than monomeric mCherry-cNLS or dimeric tdTomato-cNLS. C) Simulating RFP-cNLS propagation using the baseline parameter set (See also Supplementary Table 1.), but varying the protein half-life between 1 to 60 hours shows that varying values above 10 hours (true for each RFP-cNLS in (B)) have little effect on the predicted propagation behavior.

Figure S9



Supplementary Figure 9: Recovery of Myonuclear Domain Following Simulated Hypertrophy and Myonuclear Fusion. Simulations of RFP-cNLS diffusion and import were conducted using the initial parameter set (Supplementary Table 1). The propagation of RFP-cNLS extended following hypertrophy without the addition of myonuclei (black line to blue line), but additional myonuclei could be added (red line) to recover the original propagation profile.

Parameter	Value	Units
Nucleus Diameter	10	μm
Myotube Diameter (W)	20	μm
Myotube Half-Length	350	μm
Nucleus Spacing	25	μm
Diffusion Coefficient (D)	1	$\mu m^2/s$
Protein Half-life	30	hours
Import Coefficient (I)	1	molecules/NPC/s/μM
NPC Density	12	$NPCs/\mu m^2$
Coefficient for Passive Transport across NPC (D_N)	0	molecules/NPC/s/μM

Supplementary Table 1: Initial parameter set for mathematical model of RFP-cNLS transport.

Supplementary References

1. J. L. Ruas, L. Poellinger, T. Pereira, Role of CBP in regulating HIF-1-mediated activation of transcription. *J Cell Sci* **118**, 301-311 (2005).