

Supplemental Information

Nucleolin-Mediated RNA Localization Regulates

Neuron Growth and Cycling Cell Size

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Supplementary Material for:

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Extended Experimental Procedures

Animals. The study was conducted in accordance with the guidelines of the Weizmann and USC Institutional Animal Care and Use Committees (IACUC). Adult (8-12 weeks old) male rats were purchased from Harlan Laboratories. All the mouse strains were bred and maintained at the Veterinary Resources Department of the Weizmann Institute. For live imaging strains of interest were cross-bred with a transgenic line expressing YFP in sensory neurons (Feng et al., 2000).

Reagents, antibodies and immunofluorescence. Synthetic DNA aptamers with or without 5'-FITC or Cy3 labeling (AS1411 – GGTGGTGGTGGTTGTGGTGGTGGTGG; Control CCTCCTCCTCCTTCTCCTCCTCCTCC) were from IDT (Jerusalem, Israel). Rabbit anti-NF-H was from Chemicon (AB1989); mouse anti-NF-H clone N52 was from Sigma; Rabbit anti-GFP was from Abcam (ab6556). Rabbit anti Nucleolin was from Abcam (ab50279); Rabbit anti Dynein Heavy chain antibodies were from Santa Cruz (SC-9115) or from Proteintech (DYNC1H1, # 12345-1-AP); Importin β 1 antibodies were either a mouse monoclonal generated in-house (clone 73, raised against full length recombinant importin β 1) or a rabbit polyclonal from MyBioSource (MBS713065). Rhodamine-phalloidin was from Invitrogen (R415). Anti-puromycin was from Millipore (MABE343, clone 12D10). DAPI (Sigma D8417) was used for nuclear staining at 5 μ g/ml. Fluorescent secondaries were from Jackson ImmunoResearch. Cultured DRG neurons were fixed with 4% paraformaldehyde for immunostaining. Sciatic nerve segments were fixed in 4% paraformaldehyde, frozen, and sectioned at 12 μ m thickness. Growth cones and axon tips imaging was on a Nikon Ti-LAPP illumination system using an Andor EMCCD camera. The images were captured at X40 magnification with a long distance Nikon super plan fluor ELWD objective (NA 0.6) and analyzed by NIS-ELEMENTS software. Other imaging was in an Olympus FV1000 Confocal laser-scanning microscope at X40 magnification with oil-immersion Olympus UPLSAPO objective (NA 1.35) and analyzed by FV10-ASW2.0 software. For visualization of DAPI, Cy2, Rhodamine RED-X, and Cy5, we used 350, 489, 543 and 633 nm wavelengths, respectively. IRDye 800CW Donkey anti Rabbit was used as the secondary antibody for fluorescent quantification of Western blots, and blots were imaged on an Odyssey Fc imaging system (Li-COR).

DRG neuron cultures. DRG culture preparations were as previously described (Hanz et al., 2003; Perlson et al., 2005; Rishal et al., 2010). Briefly, adult rat or mouse DRGs were dissociated for neuron cultures with 100 U of papain followed by 1 mg/ml collagenase-II and 1.2 mg/ml dispase. The ganglia were then triturated in HBSS, 10 mM Glucose, and 5 mM HEPES (pH 7.35). Neurons were recovered through percoll, plated on laminin, and grown in F12 medium. For compartmentalized cultures neurons were plated in Boyden chambers with poly-L-lysine/laminin coated porous membranes (BD Falcon, Bedford, MA) as previously described (Willis and Twiss, 2011).

Neuronal growth rate analyses. DRG neurons from the indicated conditions and genotypes were imaged every hour during continuous culture in a Fluoview (FV10i, Olympus) automated confocal laser-scanning microscope with built-in incubator chamber, or in an ImageXpress Micro (Molecular Devices) automated microscopy system. Neuronal morphology was quantified using WIS-Neuromath (Rishal et al., 2013) or MetaXpress (Molecular Devices). Visualization of axonal projections in E11.5 mouse forelimbs was carried out by NFH immunostaining in whole mount, followed by quantification with WIS-Neuromath.

Fluorescence In-Situ Hybridization (FISH) and immunostaining on cultures. Antisense oligonucleotide probes for Importin β 1 mRNA were designed using Oligo 6 software and checked for homology and specificity by BLAST. cRNA probes for GFP reporter mRNA in transfected neurons were as previously described (Vuppalanchi et al., 2010). Hybridization to DRG neuronal cultures was as previously described (Willis et al., 2007). For colocalization of RNA and protein, fluorescently-labeled 'Stellaris' RNA probes were used (BiosearchTech) as previously described (Spillane et al., 2013). Hybridization to tissue sections was performed as previously published (Muddashetty et al., 2007), with minor modifications. Briefly, sciatic nerve was fixed for 2 hr in 2% paraformaldehyde. After overnight cryoprotection in 30% sucrose at 4°C, nerves were processed for cryosectioning with 10 μ m thick sections laid onto *Superfrost^{plus}* glass slides (Fisher). Cryostat sections were stored at -20°C until used. Sections were warmed to room temperature, rinsed in PBS for 20 min and then soaked in 0.25% acetic anhydride, 0.1 M triethanolamine hydrochloride, and 0.9% NaCl at pH 8.0 for 10 min. Sections were then rinsed in 2X SSC, dehydrated through a graded series of ethanol (70%, 5 min; 95%, 5 min; and 100%, 5 min), delipidated in chloroform for 5 min and rinsed with 100% ethanol. Sections were washed in 1X SSC and then hybridized overnight at 42°C with 1.2 ng/ μ l digoxigenin labeled probes in 2X SSC plus 40% formamide, 250 μ g/ml tRNA, 100 μ g/ml sheared salmon sperm DNA, and 10% dextran sulfate. Sections were washed in 1X SSC three times for 15 min each

at 50°C, PBS for 5 min, and PBS with 0.3% Triton X-100 for another 5 min. For immunodetection sciatic nerve sections used for *in situ* hybridization were equilibrated in 'IF buffer' (1% heat-shock BSA, 1% protease-free BSA [Roche], 50 mM Tris, and 150 mM NaCl) and then blocked in IF buffer containing 2% heat-shock BSA and 2% fetal bovine serum for 1 hour. After blocking, samples were incubated for 2 hours at room temperature in the following primary antibodies diluted in IF buffer: chicken anti-neurofilament H (1:1,000; Chemicon), chicken anti-neurofilament M (1:1,000; Aves Laboratories), chicken anti-neurofilament L (1:1,000; Aves Laboratories) and rabbit anti-S100 (1:500; Dako). Samples were washed in IF buffer three times and then incubated for 1 hour in the following secondary antibodies diluted in IF buffer: Cy5-conjugated anti-rabbit, FITC-conjugated anti-chicken, and Cy3-conjugated mouse anti-digoxigenin antibodies (1:200 each; Jackson ImmunoRes.). To increase sensitivity of detecting hybridizations in the tissue sections, an additional Cy3-conjugated anti-mouse IgG F(ab') fragments antibody (1:200; Jackson ImmunoRes.) was included with secondary antibody incubation. Coverslips were mounted with gelvatol with 6 mg/ml *n*-propyl gallate (Bassell et al., 1998). Tissues were imaged using a Leica TCS/SP2 confocal system on an inverted Leica DMIRE2 microscope. Pearson's coefficient for potential colocalization of mRNA and protein signals was calculated from confocal image stacks of Stellaris FISH/IF using Volocity software (Perkin Elmer).

Capillary Electrophoresis Immuno-quantification. Automated capillary electrophoresis immuno-quantification was carried out on a Wes instrument (ProteinSimple, San Jose, USA). Runs were conducted as described (Harris, 2015), using the default parameters specified by the manufacturer, except for an increase in matrix loading to 18 sec. For quantification of immunoprecipitates (IP), undiluted eluates from the IP procedure were combined with Wes fluorescent master mix and heated for 5 min at 95°C. Anti-kinesin heavy chain antibody was diluted 1:50. All other reagents (antibody diluent, secondary antibodies) were from ProteinSimple. Analyses were performed on 15 or 30 sec image exposures.

Fluorescence Recovery after Photobleaching (FRAP). Dissociated DRG cultures from adult Sprague Dawley rats were transfected with myr-eGFP-3'UTR plasmids prior to plating using an Amaxa Nucleofection system. FRAP was performed using a Leica TCS-SP2 confocal microscope fitted with an environmental chamber to cells at 37°C, 5% CO₂. Forty-eight to seventy-two hours after transfection, GFP-expressing neurons were chosen for FRAP analyses. 40× oil immersion objective (numerical aperture = 0.7) was used for imaging with the pinhole of the confocal set to 4 airy units to ensure that the entire thickness of axons (2–4 μm diameter)

was exposed to laser emission. All experiments employed the 488 nm laser line for GFP excitation and photobleaching with energies as indicated below. GFP emission was collected with a band filter set to 498–530 nm with PMT energy, offset, and gain matched for all collection sets. Prior to photobleaching, neurons were imaged every 30 s for 15 min with 15% laser power. A region of interest (ROI) of the terminal axon was then exposed to 75% laser power for 40 frames at 3.2 s intervals. Recovery of GFP emission was then monitored every 30 s over 30 min using 15% laser power. The raw data from multiple time-lapse experiments was used to calculate from matched images for the bleached ROI. In some experiments, cultures were pretreated with 50 μ M anisomycin immediately before imaging (i.e., at “prebleach”) as indicated. Image processing and analysis was performed using ImageJ software (NIH, Bethesda, MD). To calculate the mean fluorescence intensity within an ROI that encompassed the terminal axon with growth cone, total fluorescence intensity of the ROI for each time was divided by overall area of the ROI. The percentage of fluorescence recovery at each time point after photobleaching was then normalized to the baseline of the mean fluorescence intensity that had been measured within the ROI of the very first image after photobleaching (0 min) and averaged for all FRAP analyses in a transfection or a treatment. For each construct tested, FRAP was analyzed on at least three neurons per well and repeated over two transfection runs. Data was analyzed using GraphPad Prism 4 software package (San Diego, CA). Two-way ANOVA was used to compare the time for the recovery between transfections and between treatments followed by Bonferroni post hoc multiple comparisons. All values were expressed as mean \pm SEM and significance was set at $p < 0.05$.

Axoplasm pull-downs and proteomics analyses. Axoplasm from rat or mouse sciatic nerve was extracted as previously described (Rishal et al., 2010), and RBP pull-downs were carried out as described (Doron-Mandel et al., 2015). Bovine axoplasm was extracted by the same procedure, using sciatic nerve dissected from fresh bovine carcasses within 20 minutes of slaughter. Streptavidin magnetic beads were washed several times with different concentrations of NaCl, and were then incubated with 100 μ M of the different RNA probes, except for the no probe sample that was incubated with water. All samples were incubated for 1 hour at 4°C. After washing the resin, 0.5 mg of rat axoplasm extract or 10 mg bovine axoplasm extract was applied to the no probe resin for 30 minutes to deplete unspecific proteins and then the unbound fraction was added to the specific probe resin for another 30 minutes. After intensive washing, bound material was eluted from the resin using SDS sample buffer. The samples from the bovine axoplasm pull-down were loaded into 10% SDS-PAGE gels, followed by Colloidal Blue staining, in-gel tryptic digest and mass spectrometry. Briefly, SDS and CBB were removed with

a 25 mM ammonium bicarbonate solution in water: acetonitrile 1:1; disulfide bridges were reduced with DTT and alkylated with iodoacetamide. After the removal of the reagent excess the proteins were digested with side-chain protected porcine trypsin (Promega) at 37°C, for approximately 4 hrs. The resulting peptides were extracted, concentrated and subjected to LC/MS/MS analysis on a linear ion trap - Fourier transform ion cyclotron resonance hybrid mass spectrometer, LTQ FT(ICR) (Thermo Scientific) directly linked to a nanoACQUITY UPLC system (Waters). The peptide fractionation was performed on a C18 column (75µm x 150 mm) at a flowrate of ~400 µl; solvent A was 0.1% formic acid in water, solvent B was 0.1% formic acid in acetonitrile. A linear gradient was applied from 2% to 35% organic in 40 minutes. Data were acquired for 60 min following sample injection, in a data-dependent manner. The 6 most abundant multiply charged ions of the MS survey were selected for MS/MS analysis; dynamic exclusion was enabled. Precursor ions were measured in the magnet, CID experiments were performed in the linear trap. Peaklists were generated using the in-house software PAVA, database searches were performed using Protein Prospector v.5.4.2 on the UniProtKB.2009.12.15 database was searched – each sequence belonging to *Homo sapiens*, *Bos taurus* or *Sus scrofa* was also randomized and added to the database. Eventually 239070/21095996 entries were searched. Only tryptic peptides were considered, 1 missed cleavage was permitted. Mass accuracy 20 ppm and 0.8 Da, for precursor ions and CID fragments, respectively. Fixed modification: carbamidomethyl Cys; variable modifications: Met oxidation, cyclization of N-terminal Gln, and acetylation of protein N-termini; 2 such modifications/peptide were permitted. *Acceptance criteria*: Minimum scores 22 and 15, for proteins and peptides, respectively. Maximum E-values: 0.05 and 0.1, for proteins and peptides, respectively. Homologous proteins that were identified from at least 1 unique sequence were retained for further analyses.

Quantitative mRNA Analysis by Real-Time PCR. QPCR was performed as previously described (Nilsson et al., 2005). Briefly, axonal or soma RNA was extracted from Boyden chamber inserts using Trizol (TRI, Sigma-Aldrich) according to the manufacturer's protocol. RNA was reverse transcribed (Superscript III, Invitrogen) and Real-Time PCR was performed using Taqman (Applied Biosystems) primer kits for β -actin (normalization control) and Importin β 1, or perfecta SYBR green (Quanta biosciences) and gene specific primers for Importin β 1, Kif5A, Kif5B and 18S RNA.

Fibroblast cultures and FACS analyses. NIH 3T3 cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma) at 37°C in 5% CO₂. Cells were harvested with PBS and incubated with 10 µg/ml Hoechst 33342 (Sigma, B 2261) at 37°C for 45 minutes and 5 µg/ml Propidium Iodide (Molecular probes, P-1304) for flow cytometry analyses on a LSRII cell analyzer, collecting 30,000 events per sample. Data was processed using FCS-Express software.

Electron Microscopy. Neurons were cultured on sapphire disks and fixed 48 hr after plating using high pressure freezing (HPF) in a Bal-Tec HPM10, followed by freeze substitution, washing, embedding into HM20 resin, and ultrathin sectioning (70–90 nm). Sciatic nerves were fixed in 0.1% glutaraldehyde, washed, cut to 100 µm sections by vibrotome, then underwent high pressure freezing (HPF) in a Bal-Tec HPM10, followed by freeze substitution, washing, embedding into LR Gold resin, and ultrathin sectioning (70–90 nm). Sections were collected on nickel grids coated by formvar film. For immunostaining, grids were first reacted with anti-dynein HC1, followed by anti-Importin β1 when required for double-labeling, and secondary anti-rabbit IgG with different sizes of gold particles. Single labeling was also performed for Importin β1 and nucleolin. The grids were then stained in uranyl acetate and lead citrate and analyzed under 120 kV on a Tecnai 12 (FEI) Transmission Electron Microscope with a EAGLE (FEI) CCD camera using TIA software. Quantitative analysis was performed by Fiji (Schindelin et al., 2012), using a macro script written in-house to identify gold particles of specified diameter (8-15 nm). The macro segments dark objects by applying rolling-ball background subtraction, thresholding with fixed value and separating touching objects using watershed. Relevant regions of interest (ROI) can then be selected for analysis. All particle counts were normalized to the selected ROI area.

Proximity Ligation Assay (PLA). PLA was performed using the Duolink system (Sigma) according to the manufacturer's instructions, with the following primary antibodies: rabbit anti-Dynein HC (SC-9115, 1:50) or rabbit anti-DYNC1H1 (Proteintech, # 12345-1-AP, 1:100) and mouse anti-Importin β1 (Clone 73, 1:2000). Interactions were detected using Sigma PLA probe anti-mouse minus DUO92004, anti-rabbit plus DUO92002, and detection kits Red DUO92008 or FarRed DUO92013. Staining was done with Phalloidin rhodamine for 30 min at RT. Quantification was performed with Cell Profiler.

Puromycin labeling. Puromycilation was performed as previously described (David et al., 2012; tom Dieck et al., 2015) with minor modifications. Neurons or NIH 3T3 cells were pre-incubated with Anisomycin 40 mM (Sigma, A9789) for 30 min followed by puromycin 5 mM (Sigma, P8833) for 10 min. Imaging of neuronal puromycilation was by a Nikon eclipse - Ti-E microscope at 40X magnification using an Andor EMCCD camera. Analysis of puromycin labeling in axon tips was done by NIS-Elements software. Quantification of puromycin labeling on confocal images of 3T3 cells was done using an ImageJ macro. Peripheral staining was defined by a mask formed by a ring spanning x pixels from the plasma membrane, where x is a function of the average area per cell calculated for each image according to the formula $x = (10 * \text{Area}) / \text{Average_Area_Control_Cells}$, to correct for the possible confounding differences in periphery/total ratios in cells of different size. Puromycin staining intensity in different cytoplasmic regions was calculated after subtraction of the nuclear area.

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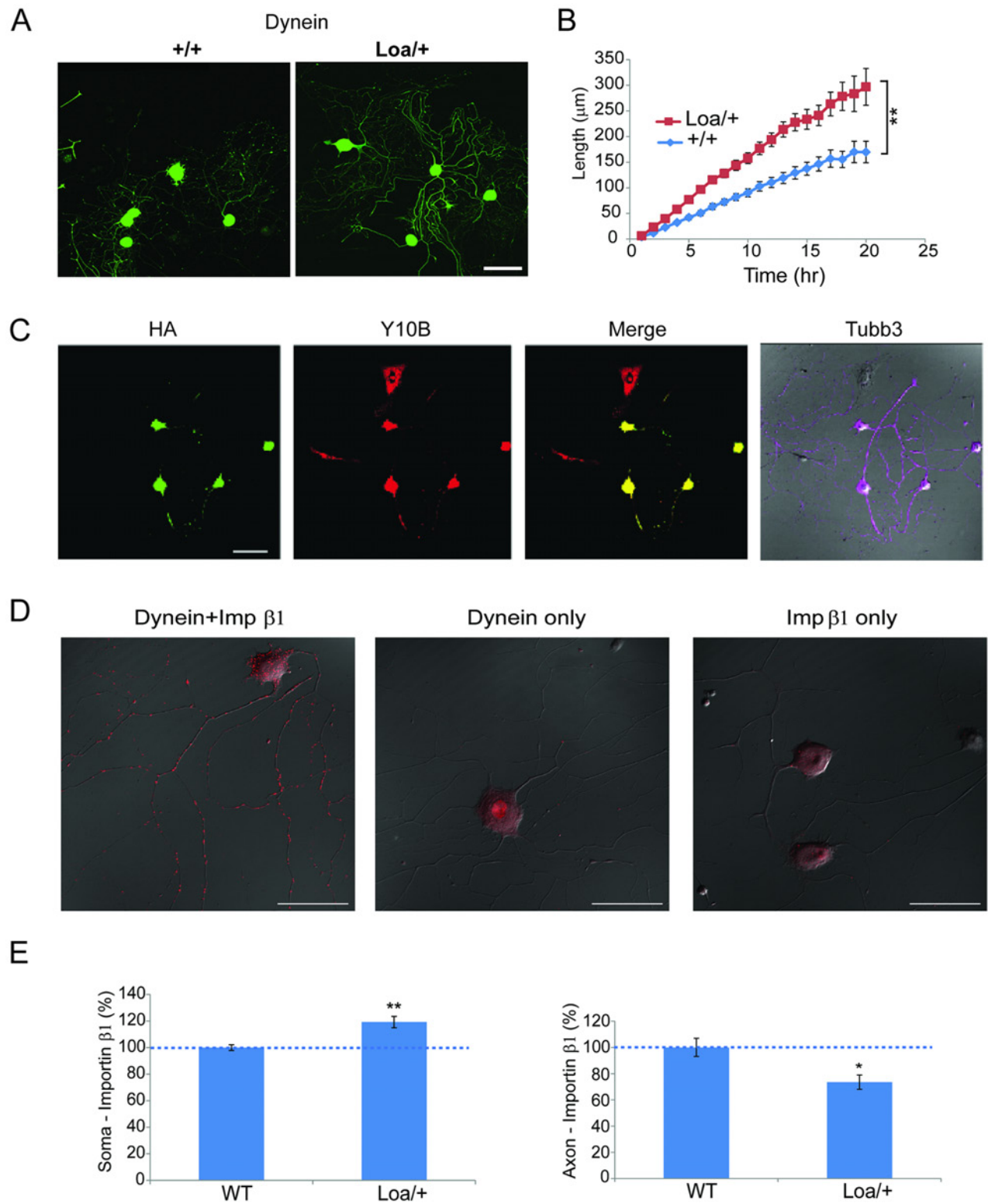
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Supplemental Figures

Figure S1 – connected to Figure 1



(A) Representative fluorescent images after 48 hr culture of YFP expressing DRG neurons from adult wild type or *Loa* heterozygote mice. Scale bar 100 μm .

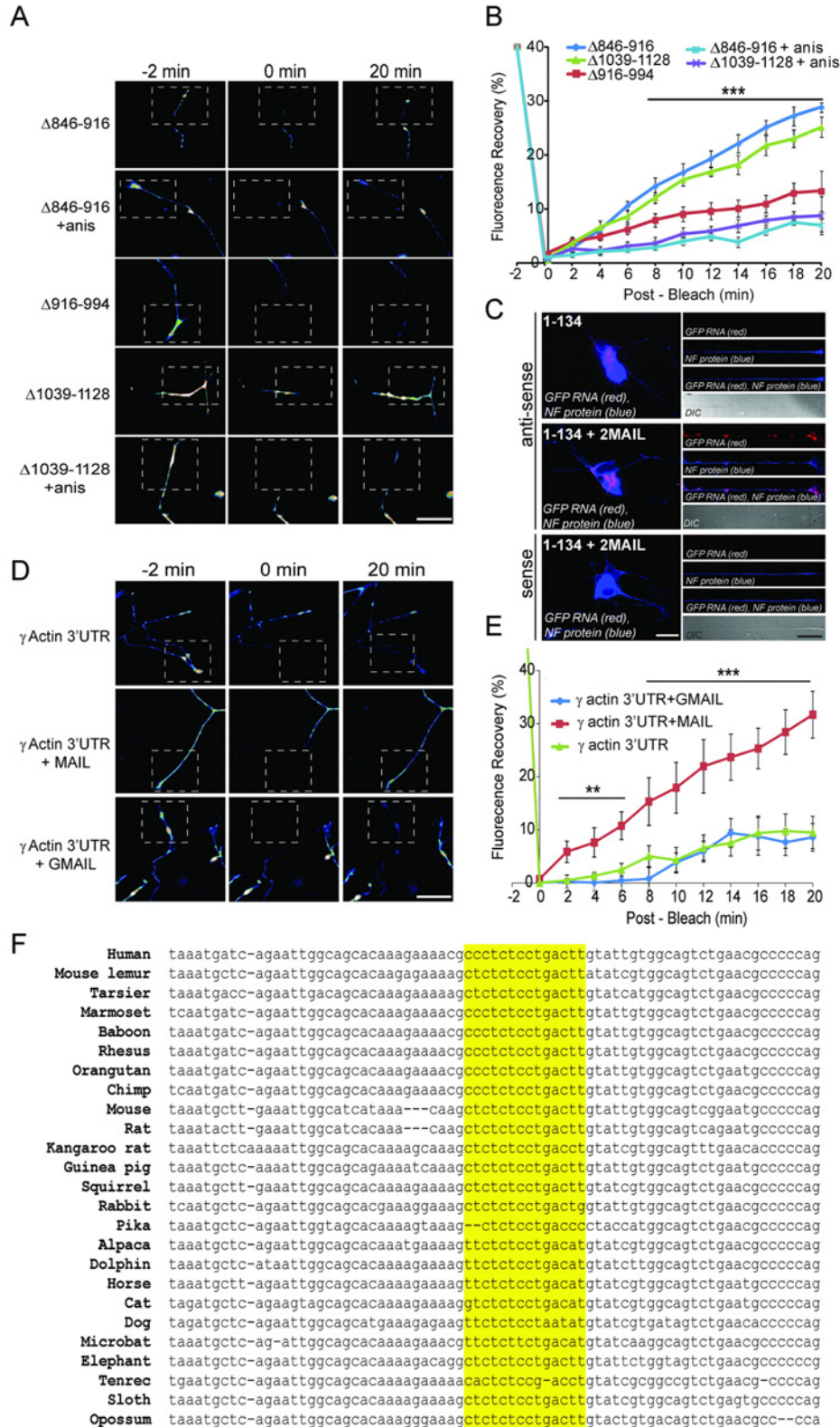
(B) Quantification of time-lapse imaging of YFP expressing DRG neurons over 48 hr in culture. Images were taken every hour in a Fluoview FV10i incubator microscope. 3x3 montages of neighboring acquisition sites were analyzed using ImageJ. Longest neurite growth rates in these experiments were $6.5 \pm 1.2 \mu\text{m/hr}$ for wild type versus $12.7 \pm 1.6 \mu\text{m/hr}$ for *Loa/+* mice, Mean \pm SEM, $n \geq 30$ cells per experimental group, ** $p < 0.01$ for comparison of growth rates (One-way ANOVA).

(C) DRG neuron cultures from *Islet1-Ribotag* mice were stained with HA, Y10B (Ribosomal RNA) and Tubb3 antibodies before confocal imaging. The HA staining is specific for axons. Scale bar 50 μm .

(D) Representative images from proximity ligation assay (PLA) to identify importin β 1-dynein complexes in DRG neurons grown for 48 hr in culture. After 48 hr the neurons were fixed with 4% PFA and stained with dynein heavy chain 1 (rabbit polyclonal) and importin β 1 (mouse monoclonal) antibodies, followed by rabbit and mouse PLA probes. After developing the reaction, imaging was carried out on a confocal microscope at 60X oil magnification. Scale bar 50 μm .

(E) Quantification of Importin β 1 protein levels in soma versus axon compartments of sensory neurons in culture reveals a significant increase in cell bodies and a decrease in axons of cultures from *Loa* heterozygote (*Loa/+*) mice as compared to wild type. DRG neurons isolated from WT or *Loa/+* mice were grown in Boyden chambers for 48 hours before protein extraction from soma and axon compartments. Importin β 1 levels in protein extracts were determined by immunoprecipitation in a Wes (ProteinSimple) automated capillary electrophoresis apparatus. Average \pm SEM, $n = 5$ per genotype *, $p < 0.05$, **, $p < 0.01$ (Student's t-test).

Figure S2 – connected to Figure 2



(A) The indicated deletion constructs of the importin β 1 3'UTR were fused with a destabilised myr-EGFP reporter and transfected to sensory neurons for FRAP analyses, with recovery monitored over 20 min. Representative images from time-lapse sequences before (-2 min) and after photobleaching (0 and 20 min) in the boxed region of interest are shown. Scale bar 25 μ m.

(B) Quantification of the FRAP analyses shown in A. Average recoveries are shown (% of pre-bleach levels \pm SEM). Anisomycin treated neurons were exposed to 50 μ M of the inhibitor prior to pre-bleach imaging. Time points with significant differences in axonal fluorescence compared to that observed in anisomycin-treated cultures are indicated by *** for $p < 0.001$ (Two-way ANOVA).

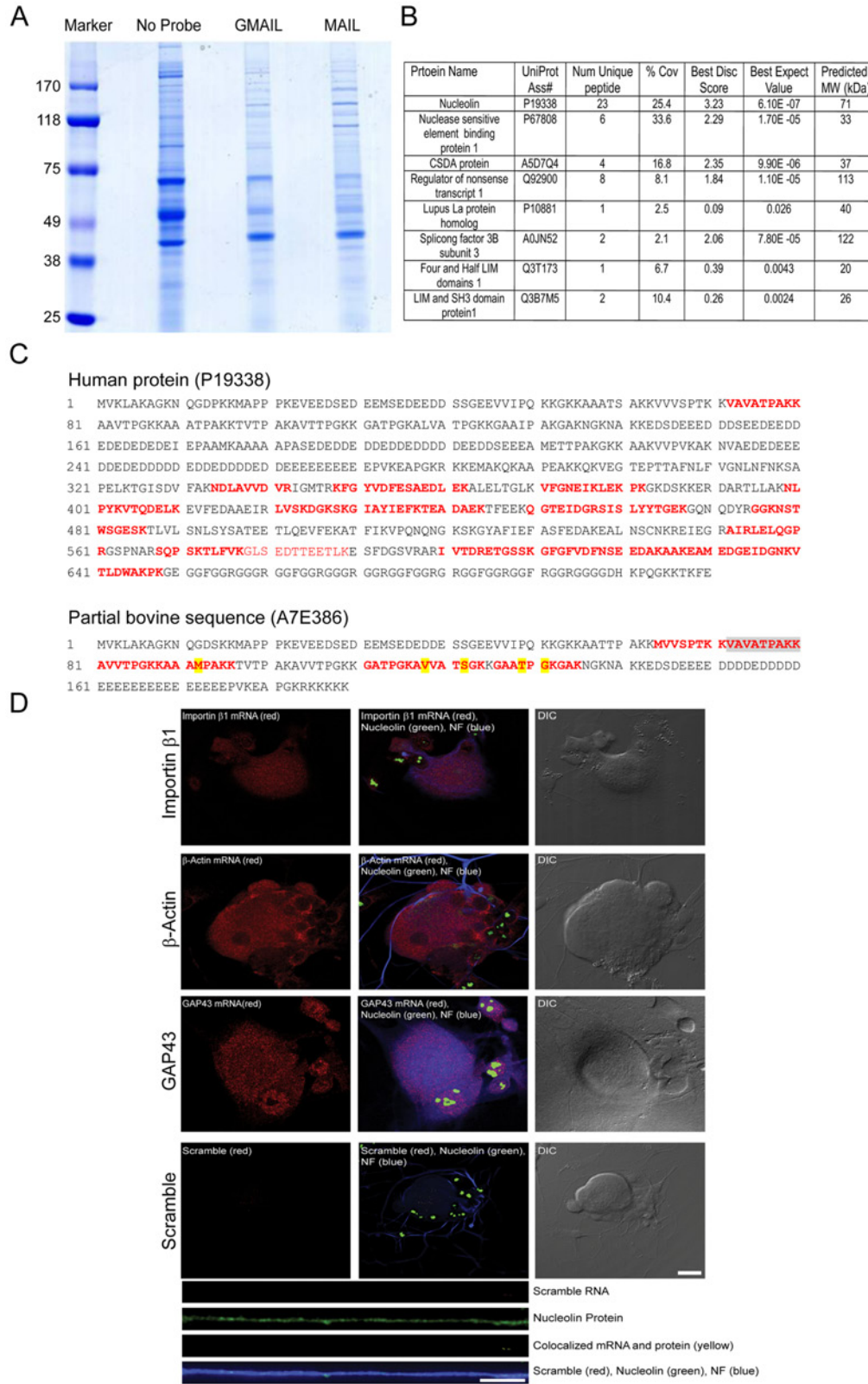
(C) In situ hybridization on neurons transfected with constructs containing the importin β 1 1-134 3'UTR region, or the 1-134 segment fused to two MAIL sequences, as indicated. Exposure matched images show that only GFP mRNA with the MAIL motif localizes into axons (right), while both reporter mRNAs are clearly expressed in corresponding cell body images (left). Sense probe hybridization for specificity control shown below. Scale bars, cell body panels 20 μ m, axon panels 10 μ m).

(D) Representative images from time-lapse sequences before (-2 min) and after photobleaching (series from 0 to 20 min) of adult DRG neurons transfected with γ actin 3' UTR constructs fused to myr-GFP. γ actin 3'UTR axonal fluorescence compared to γ actin 3'UTR fused to MAIL or to GMAIL. The boxed regions represent the area subjected to photobleaching with recovery monitored over 20 min. Scale bar 25 μ m.

(E) Quantification of the FRAP experiments shown in D over multiple time-lapse sequences. Average recoveries are shown as % of pre-bleach levels \pm SD, $n > 6$, ** denotes $p < 0.01$, *** denotes $p < 0.001$ (Two-way ANOVA).

(F) MAIL sequence conservation in importin β 1 from different species (UCSC genome browser). The loop region is highlighted in yellow.

Figure S3 – connected to Figure 3



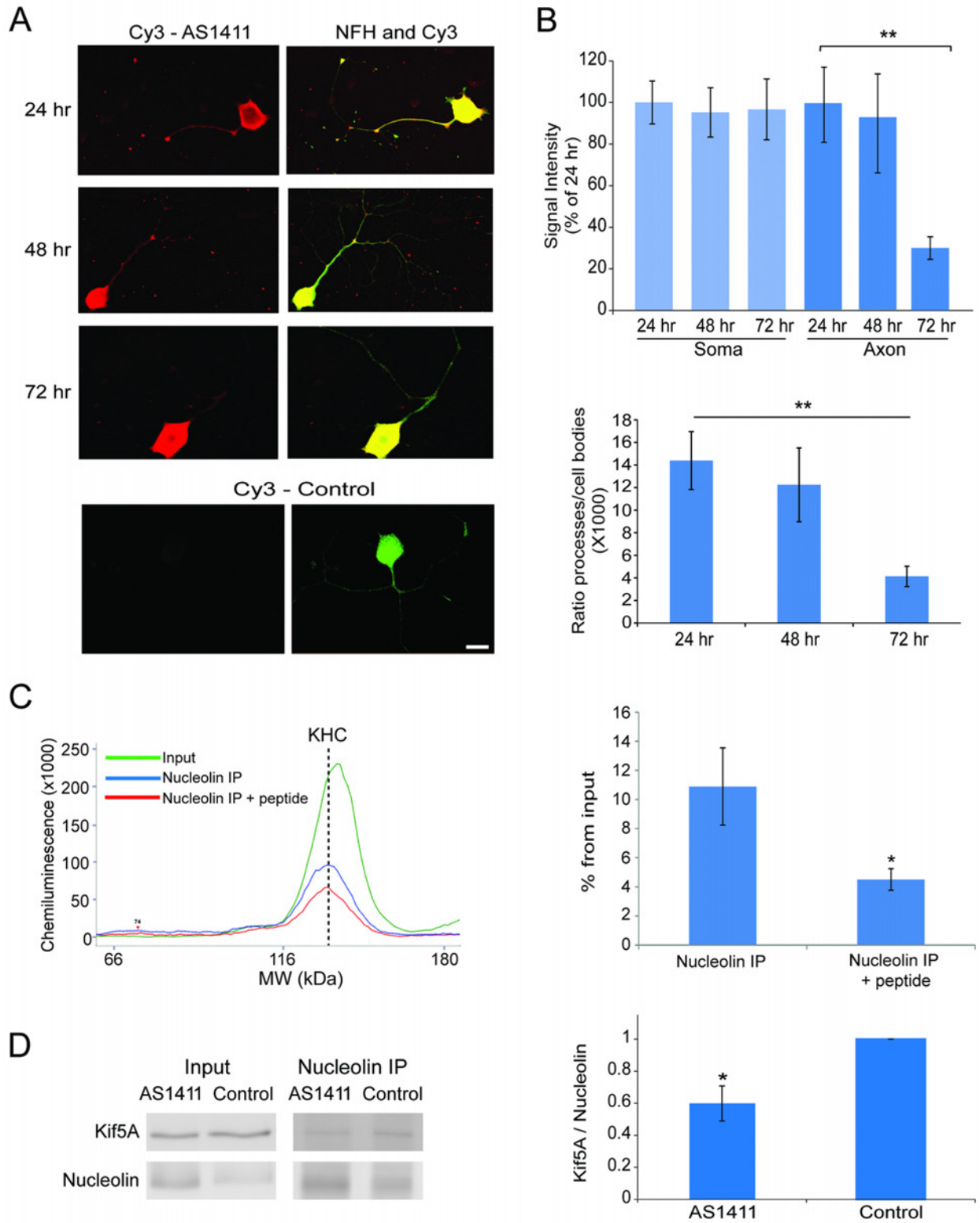
(A) 10% SDS-PAGE of proteins from bovine axoplasm bound by biotinylated MAIL or GMAIL RNA probes and precipitated on immobilized streptavidin. 10 mg bovine axoplasm extract were used as input for each pull-down lane.

(B) Proteins identified by mass spectrometry from MAIL but not control pull-downs. Protein identifications are from the UniProtKB.2009.12.15 database.

(C) Nucleolin was not detected in the two control pull-downs and was identified with a large number of unique peptides and high sequence coverage from MAIL pull-downs. The identification was mostly based on the homologous human protein sequence, since the UniProt database contains only a partial sequence for the bovine protein. 18 unique peptides were identified meeting the acceptance criteria from the pre-filtered pull-down experiment, and 23 sequences without prefiltering. Grey shading indicates an identical sequence stretch between species, while the highlights in the identified sequences indicate the amino acid differences detected between human and bovine proteins.

(D) FISH on neuronal cell bodies for importin β 1, β actin or GAP43 transcripts as indicated, or scrambled sequence control (all in red), together with immunostaining for nucleolin (green). Scrambled sequence control is shown also for axons visualized by NF immunostaining (blue). Scale bars: cell body = 10 μ m, axon = 5 μ m.

Figure S4 – connected to Figure 4



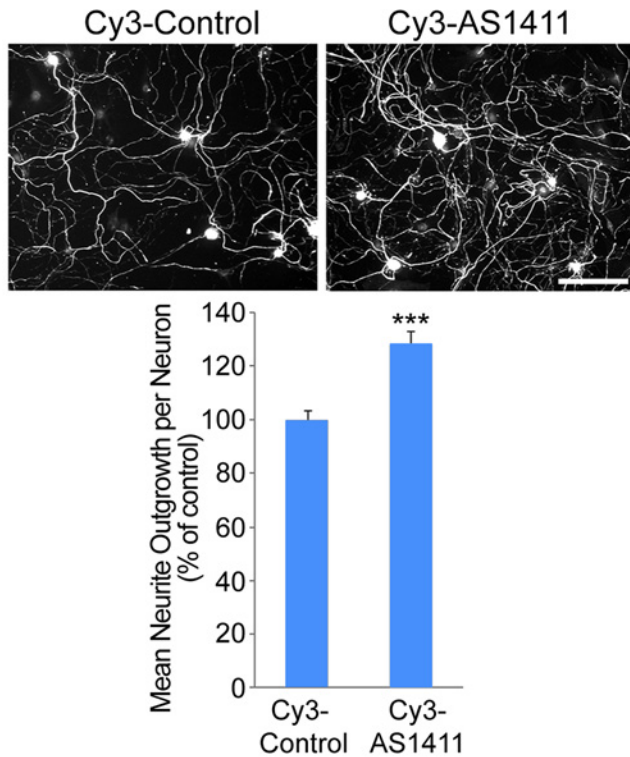
(A) Cy3 labeled AS1411 and control aptamers were added to sensory neuron cultures to a final concentration of 20 μ M. Neurons were fixed at the indicated time points and stained as indicated. Cy3 signal shown in red, NFH immunostaining in green, overlay in yellow. Scale bar 10 μ m.

(B) Quantification of soma and axon signal intensity in the experiment of Panel A 48 hr and 72 hr after AS1411 uptake. The upper graph shows quantification normalized to the signal after 24 hr, the lower graph shows the ratio of axon to cell body signal intensities. After 72 hr there is a significant decrease in cy-3 signal only in axons. Mean \pm SEM, n = 8, ** p < 0.005 (Student's t-test).

(C) Automated capillary electrophoresis quantifications of kinesin heavy chain (KHC) immunoreactivity co-immunoprecipitated with nucleolin from sciatic nerve axoplasm. Control immunoprecipitations were carried out in the presence of a nucleolin antibody blocking peptide. Representative traces of KHC immunoreactive peaks are shown on the left, and quantifications on the right. Mean \pm SEM, n=3, * denotes p < 0.05 (Paired Student's t-test).

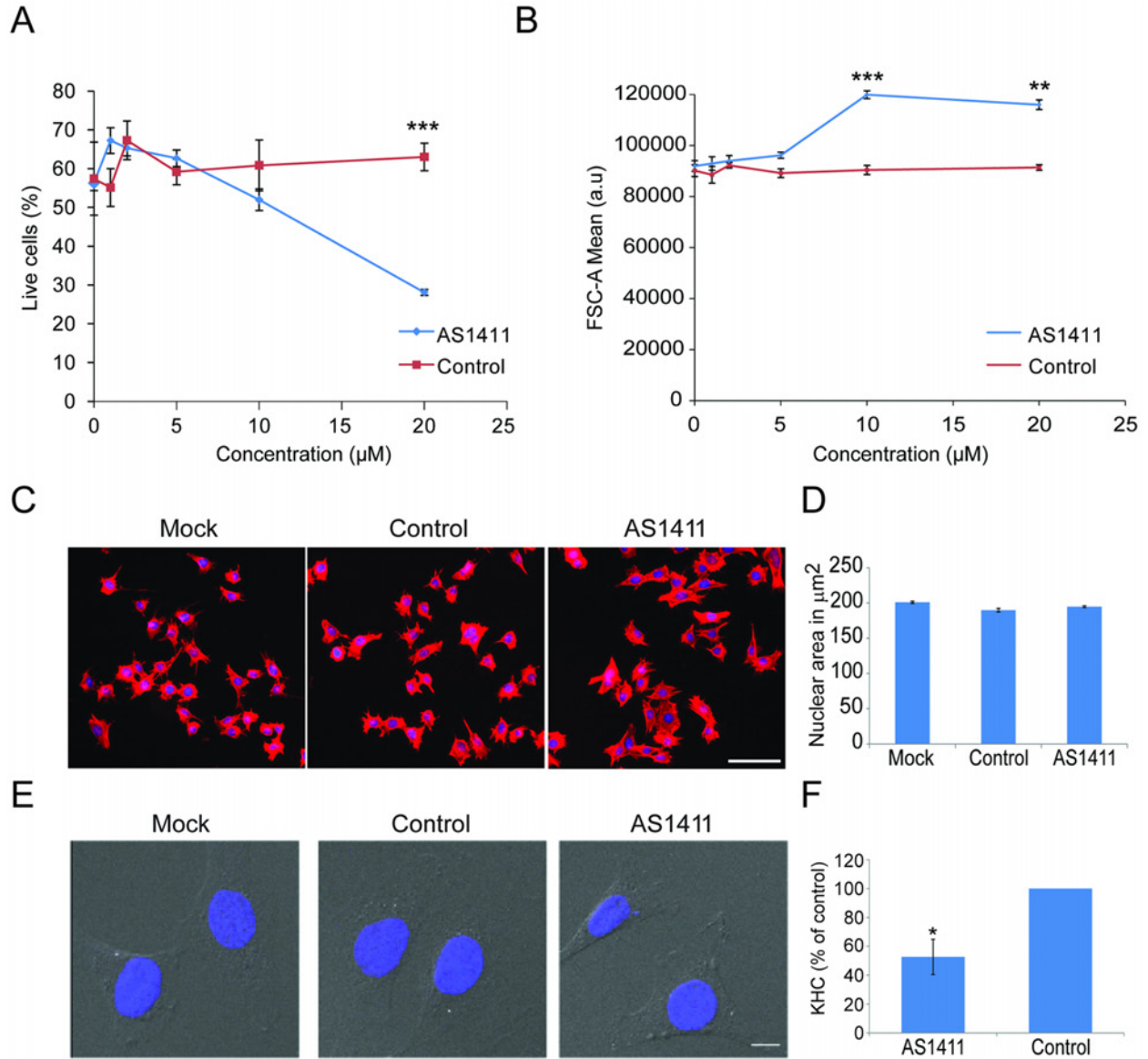
(D) Kif5A Western blots of nucleolin i.p. from DRG neurons after 48 hr in culture in the presence of AS1411 or control aptamers. The graph on the right shows quantification of co-immunoprecipitated Kif5A, mean \pm SEM, n=3 biological repeats. *, denotes p-value < 0.05 (Paired Student's t-test).

Figure S5 – connected to *Figure 5*



Photomicrographs of adult mouse DRG neuronal cultures immunostained for peripherin (EMD Millipore, AB1530, dilution 1:1000). Cultures were exposed to 10 μ M Cy3-Control or Cy3-AS1411 for two days to allow aptamer uptake. Neurons were then trypsinized, replated, and cultured for an additional three days for neurite outgrowth. Quantification of neurite outgrowth demonstrates significantly more neurite outgrowth per neuron for Cy3-AS1411-treated neurons compared with control. Data are mean \pm S.E.M. $n = 96$ wells from three separate experiments. ***, $p < 0.001$, Student's t -test. Scale bar = 200 μ m.

Figure S6 – connected to Figure 6



(A) 3T3 cells were incubated for 48 hours with different concentrations of AS1411 or control aptamer, and then harvested and incubated with 5 µg/ml Propidium Iodide for viability analysis by flow cytometry, collecting 30,000 events per sample. Mean ± SEM, n = 3, *** denotes $p < 0.001$ (Student's t-test).

(B) 3T3 cells were incubated as in Panel A and then underwent cell size analysis by flow cytometry, collecting 30,000 events per sample. Mean \pm SEM, n =3, ** denotes $p < 0.005$, *** denotes $p < 0.001$ (Student's t-test).

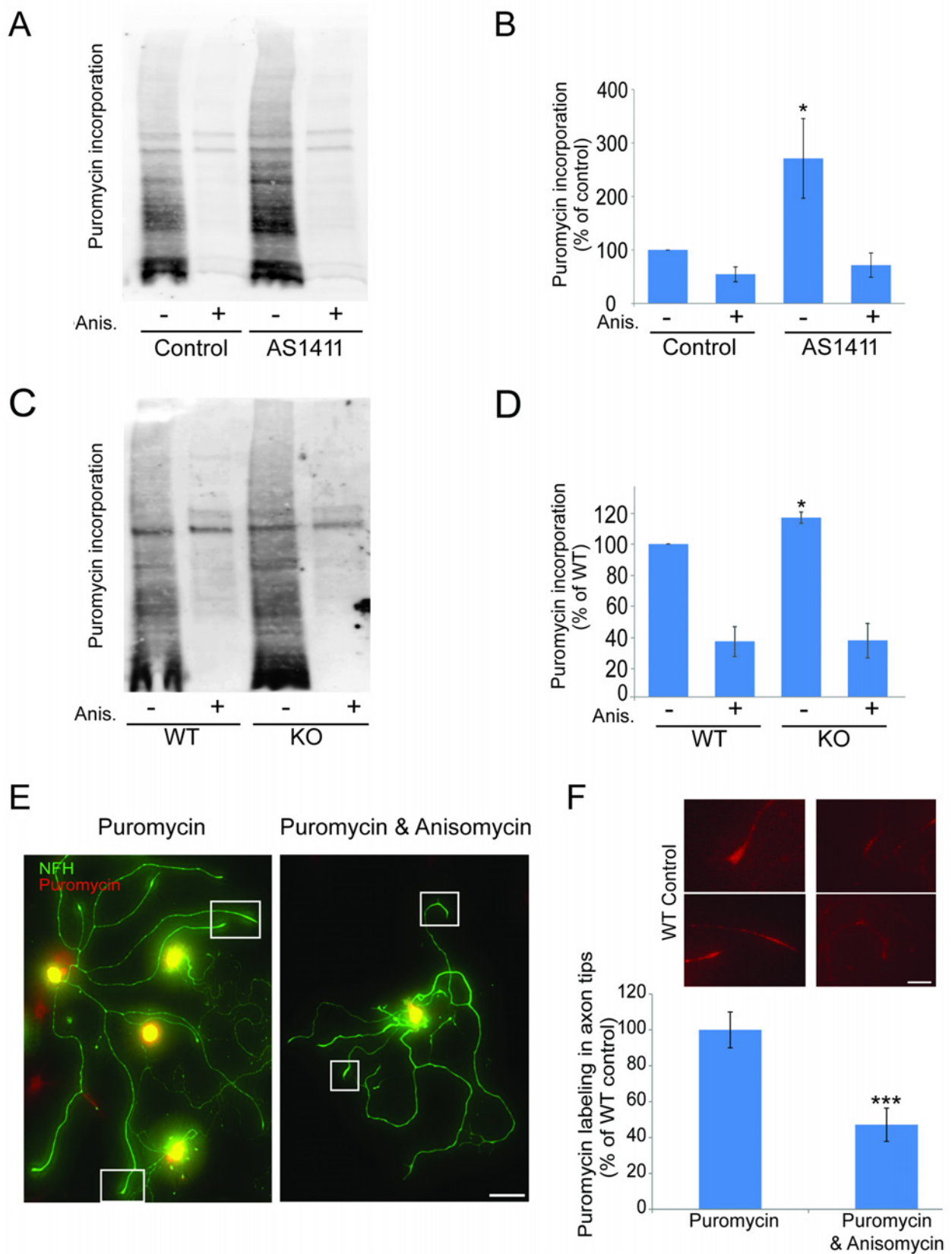
(C) 3T3 cells were incubated with 10 μ M AS1411 or control aptamer for 48 hours, after which 20,000 cells were replated for another 24 hours in fresh medium without aptamer before fixing and staining with DAPI and rhodamine-phalloidin. Scale bar 100 μ m.

(D) Quantification of nucleus area in 3T3 cells from the experiment described in Panel C reveals that aptamer treatment has no effect on nuclear area. Mean \pm SEM, n >2000.

(E) 3T3 cells cultured and treated as described in Panel C before fixing and staining with DAPI. Representative high magnification images, scale bar 10 μ m.

(F) Quantification of kinesin heavy chain (KHC) pulled down by MAIL RNA probe from 3T3 cell lysates. Cells were incubated with AS1411 or control aptamers for 48 hr in culture before lysis and pull-down. KHC levels quantified by automated capillary electrophoresis. Data shown as % from control. Mean \pm SEM, n=5, * denotes $p < 0.05$ (Paired Student's t-test).

Figure S7 – connected to Figure 7



(A) The translational activity of DRG neurons in culture was assessed by puromycin incorporation. Cultures were grown in the presence of AS1411 or control aptamer for 48 hr, and then pulsed with puromycin (5 μ M) for 10 minutes, before extraction and processing for SDS-PAGE and Western blotting with a puromycin antibody. Puromycin incorporation was validated in parallel cultures preincubated with the translation inhibitor Anisomycin (40 μ M) for 30 min before puromycilation.

(B) Quantification of puromycin incorporation shows a significant increase in overall protein synthesis in AS1411-treated cultures. Mean \pm SEM, n = 4, * denotes p < 0.05 (paired Student's t-test).

(C) Puromycin incorporation in wild type versus Importin β 1 3'UTR knockout neurons, cultured and processed as described for Panel A.

(D) Importin β 1 3'UTR knockout neurons revealed significantly higher protein synthesis levels than wild type neurons. Mean \pm SEM, n = 3, * denotes p < 0.05 (paired Student's t-test).

(E) Representative images of cultured wild type sensory neurons treated with 10 μ M control aptamer for 48 hours, and then replated and cultured for an additional 24 hours in fresh medium without aptamer. Neurons were then pulsed with 5 mM puromycin for 10 min at 37°C or preincubated with 40 mM anisomycin for 30 min followed by the 5 mM puromycin pulse, and then fixed. Fixed cultures were immunostained for NFH (green) and α -puromycin (red). Scale bar 100 μ m.

(F) Anisomycin treatment significantly reduced puromycin labeling in axon tips. Scale bar 20 μ m. Axon tip synthesis quantified as ratios of cell body values and then normalized to wild type control. Average \pm SEM, n \geq 80 cells from three independent cultures, *** indicates p < 0.001 (Student's t-test).