Supporting Information

Ying et al. 10.1073/pnas.1407462111

SI Materials and Methods

Immunofluorescence Cytochemistry. Dissociated DRG neurons were grown on coverslips for 24–72 h and fixed in 4% paraformaldehyde for 20 min, followed by permeabilization with 0.1% Triton X-100 in PBS at room temperature for 30 min. The cells were then blocked with 2% goat serum, 2% horse serum, and 1% BSA for 30 min before incubation with rabbit polyclonal, anti-Luman (1:500; characterized in ref. 1), mouse monoclonal anti–β-III-tubulin (1:1,000; Millipore), and goat polyclonal, anti-calnexin (1:50; Santa Cruz Biotechnology) antibodies overnight at 4 °C. Cells were then incubated with the secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG, DyLight 594-conjugated goat anti-mouse IgG, and DyLight 594-conjugated donkey anti-goat IgG (1:1,000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Coverslips were then mounted onto glass slides with an antifade reagent with DAPI (ProLong Gold; Life Technologies) and subjected to fluorescence microscopic analysis.

Quantification of Fluorescence Signal. Alterations in axonal levels of Luman immunofluorescence signal were assessed using Northern Eclipse version 7.0 software (Empix Imaging). Axonal regions were defined and fluorescence signal over the entire area of the defined regions was measured and is expressed as average Gy/μm^2. Values were then normalized against the mean value of the average Gy/μm^2 readings for the control condition.

Immunoprecipitation. Antibodies were cross-linked to Dynabeads protein A (Invitrogen; Dynal) according to the manufacturer's instructions. Cell lysates were precleared with IgG Dynabeads protein A for 30 min at 4 °C before incubation with antibody-linked Dynabeads overnight at 4 °C. The immunoprecipitated Dynabead complexes were washed three times with RIPA buffer. Proteins were eluted by boiling in protein loading buffer and then processed for Western blot analysis.

Western Blot Analysis. Protein levels of Luman and importin-α were determined by immunoblot analysis from protein samples prepared from either freshly dissected tissues or cultured cells. Briefly, cells were lysed in RIPA buffer containing a protease inhibitor mixture. Lysates were centrifuged for 10 min at 14,000 × *g*, and supernatants were collected and protein concentration was determined by the Bradford assay method. Twenty micrograms of protein was electrophoresed on a 12% SDS polyacrylamide gel and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with blocking buffer (LI-COR Biosciences) at room temperature for 1 h and incubated overnight at 4 °C with rabbit anti-Luman (1:1,000) and mouse anti-importin-α (1:1,000). Membranes were then washed with PBST (PBS containing 0.1% Tween 20) and incubated with IRDye 800CW goat anti-mouse secondary antibody and IRDye 680LT goat anti-rabbit secondary antibody (1:10,000; LI-COR Biosciences) for 1 h at room temperature. After washing with PBST, proteins were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences). GAPDH (1:1,000; Novus) and Lamin B levels (1:200; Santa Cruz Biotechnology) were used as loading controls. For quantification, the density of each band on the immunoblot was estimated by densitometry and normalized to the density of the loading control band in the sample.

Immunofluorescence Histochemistry. Sections were washed three times for 10 min each in 0.1 M PBS and blocked with SEA BLOCK Blocking Buffer (Thermo Scientific) for 1 h at room temperature. Tissues were incubated with the primary antibodies rabbit polyclonal anti-Luman (1:500), mouse monoclonal anti–importin-β (1:200; Thermo Scientific), mouse monoclonal anti–β-III-tubulin (1:1,000; Millipore), and rabbit monoclonal anti-GRP78 (1:200; Cell Signaling Technology) overnight at 4 °C. Sections were then washed three times for 10 min each in PBS and incubated with the secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG, DyLight 594-conjugated goat anti-mouse IgG (1:1,000; Jackson ImmunoResearch Laboratories), and Alexa Fluor 350-conjugated donkey anti-rabbit IgG (1:500; Life Technologies) for 1 h at room temperature. Slides were coverslipped with antifade solution (ProLong Gold; Life Technologies).

Reverse Transcription–PCR. Total RNA was extracted using the RNeasy Kit (Qiagen) and converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The primers used in the RT-PCR were the following: Luman: 5′-TGTGCCCGCTGAGTATGTTG-3′ and 5′-AGAAGGTCGGAGCCTGAGAA-3′; γ-actin: 5′-CAACAGCAGACTTCCAGGATTTC-3′ and 5′-TTCCCAACTCAAGGCAAGTAACAAC-3′; and β-actin: 5′-CCGTAAAGACCTCTATGCCAACA-3′ and 5′-CGGACTCATCGTACTCCTGCT-3′. The veracity of the products of the RT-PCRs was confirmed by nucleotide sequencing.

Chloramphenicol Acetyltransferase Assay. Vero cells were cultured in DMEM supplemented with 10% newborn calf serum or 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in humidified air with 5% CO_2. One day before transfection, Vero cells were seeded into six-well plates at a concentration of 5×10^5 per well. One microgram of pcDNA3.1, pcLuman, pcRatLuman, or pcGFP-Lu-RFP was cotransfected with 100 ng of pCAT3BATF6 using Lipofectamine 2000 (Life Technologies). Chloramphenicol acetyltransferase (CAT) activity was measured 24 h posttransfection using the CAT ELISA Kit (Roche).

1. Lu R, Misra V (2000) Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene *trans*-inducing factor), in herpesvirus latency. *J Virol* 74(2): 934–943.

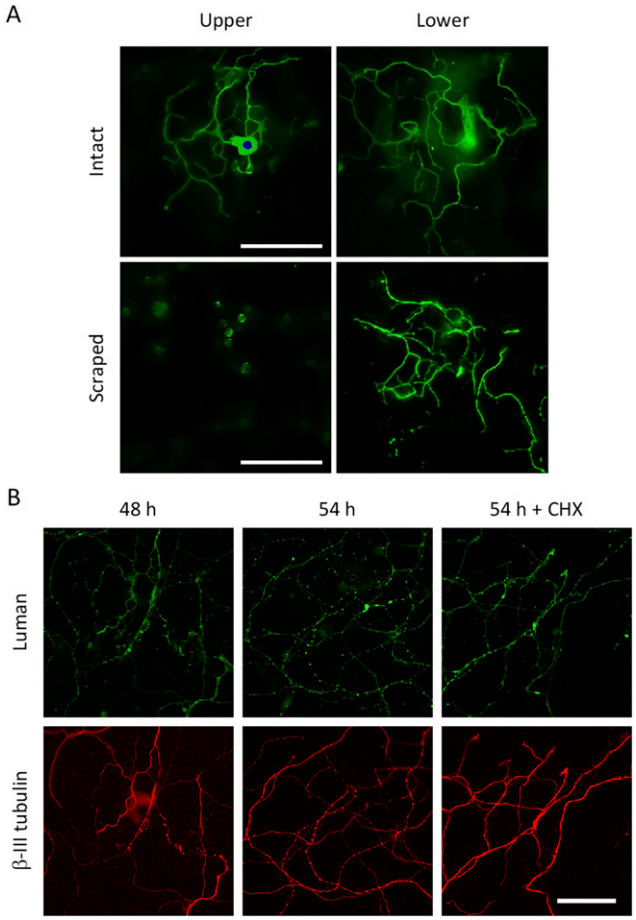


Fig. S1. Purity of axon preparations and assessment of Luman synthesis in axon preparations from naïve sensory neurons. (*A*) DRG neurons plated onto a Transwell insert 24 h before processing for immunofluorescence to detect β-III-tubulin (green) with DAPI-stained nuclei (blue). Lower-membrane images acquired directly below the upper-membrane focal plane reveal no detectable nuclear DAPI signal along the lower-membrane surface. To isolate axons, the upper membrane was scraped, removing all cellular elements from the upper-membrane surface, whereas axons that had traversed the membrane remain adhered to the lower underside surface of the membrane. (Scale bars, 100 μm.) (*B*) Naïve DRG neurons were plated onto a Transwell insert for 48 h. Upper membranes (cell bodies and proximal axons) were scraped, and the lower components (desomatized neurites) continued to be cultured ± the translation inhibitor cycloheximide (10 μg/mL) for 6 h. Luman protein levels were assessed by immunofluorescence (green), with axons identified with anti–β-III-tubulin (red). Note the lack of apparent Luman synthesis in the naïve neuron-desomatized neurites. (Scale bar, 50 μm.)

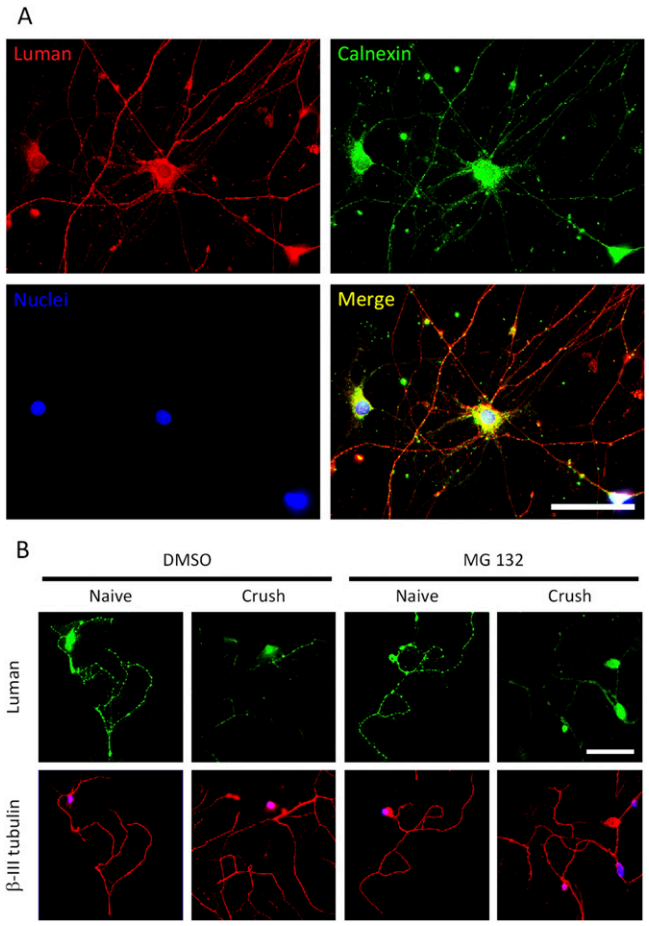


Fig. S2. Luman localizes to the ER and disappears from axons following injury. (*A*) Naïve L4,5,6 DRG neurons were cultured for 24 h on a coverslip and then processed for immunofluorescence to detect Luman (red) and the ER integral protein calnexin (green) with DAPI-stained nuclei (blue). Merge: Luman co-localizes with calnexin and appears as discrete punctae along the axons/neurites. (*B*) Dissociated L4,5,6 DRG neurons that were either naïve or injury-conditioned for 24 h before culturing for 24 h were processed for immunofluorescence to detect Luman (green) and β-III-tubulin (red), with nuclei visualized by DAPI staining (blue). Note: Axon-localized Luman has largely disappeared from the preinjured axons relative to naïve control axons. This change is not inhibited by addition of the proteasome inhibitor MG-132 (5 μM) nor altered in the presence of MG-132's diluent dimethyl sulfoxide (DMSO). (Scale bar, 100 μm.)

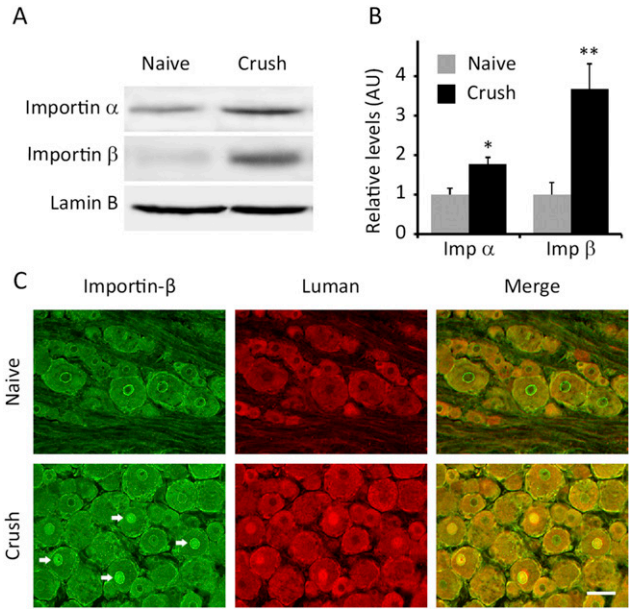


Fig. S3. Nerve injury activation of the importin system: colocalization with nuclear Luman. (*A*) Representative Western blot analysis of importin-α and -β in proteins extracted from 10-mm nerve segments just proximal to a 24-h sciatic nerve crush site with nuclear envelope protein Lamin B as the loading control. (*B*) Quantification of results performed as in *A*. Relative changes in importin-α (imp α) and importin-β (imp β) expression in response to injury as normalized to naïve levels in arbitrary units. Expression of importin-α and -β is significantly increased in response to nerve injury (mean ± SEM; $*P < 0.05$; $**P < 0.01$; $n = 3$ separate experiments). (*C*) L4 DRG sections obtained from naïve or 24-h injury-conditioned animals and processed for immunofluorescence to detect importin-β (green) and Luman (red). They reveal that 24 h after nerve injury, neuronal nuclei with increased importin-β staining (examples are indicated by arrows) also show increased nuclear Luman signal, whereas there is no nuclear Luman detected in sections from naïve animals. (Scale bar, 50 μm.)

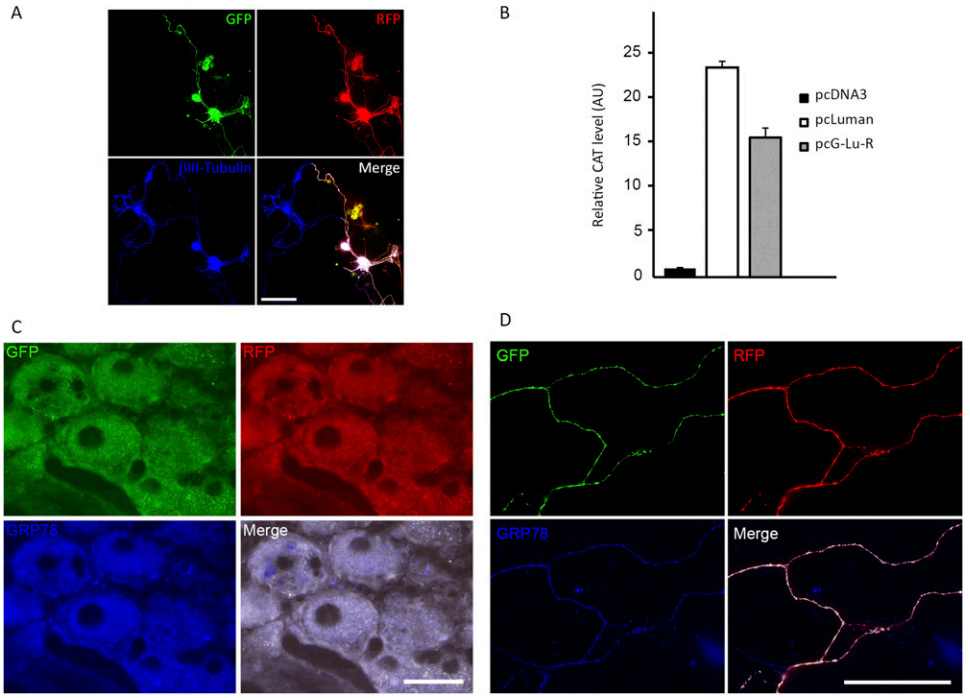


Fig. S4. Ad/GFP-Lu-RFP delivers functional GFP–Luman–RFP into DRG neurons that localizes to the neuronal ER. (*A*) DRG neuronal cultures were infected with Ad/GFP-Lu-RFP at an MOI of 100, and neurons were visualized by β-III-tubulin immunostaining (blue). (Scale bar, 100 μm.) (*B*) Luman (pcLuman) and GFP–Luman–RFP fusion protein (pcG-Lu-R) can activate CAT reporter gene expression (fold change of CAT levels in arbitrary units relative to the control plasmid) via the unfolded protein response element, but the control plasmid (pcDNA3) does not. Mean ± SEM. (*C* and *D*) DRG sections from animals infected in vivo (*C*) and DRG neurons infected in vitro (*D*) demonstrate colocalization of GFP–Luman–RFP fusion protein and ER protein GRP78. Merged images reveal that GFP–Luman–RFP fusion protein localizes to the ER both in vivo and in vitro. (Scale bars, 50 μm.)

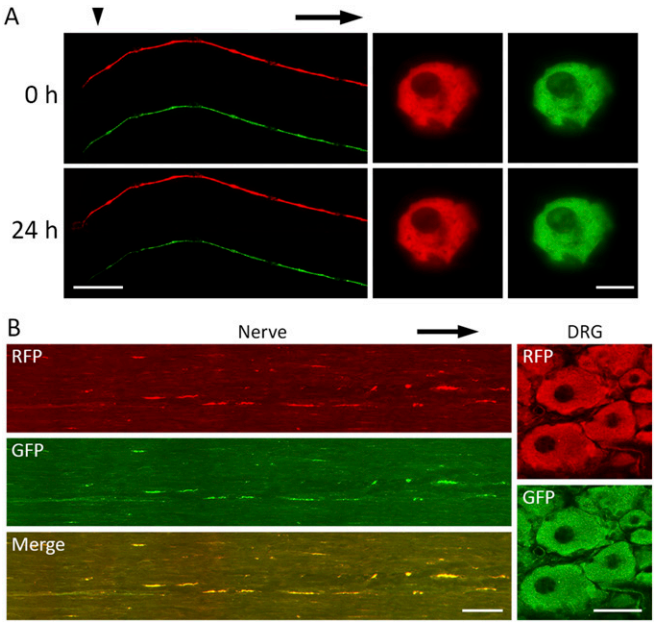


Fig. S5. Addition of NLS peptide effectively competes with the ability of importin-α to retrogradely transport cleaved Luman. (*A*) The same experiments as in Fig. 3*A*, but NLS peptide was added 24 h before the in vitro injury. [Scale bars, 50 μm (axon) and 20 μm (cell body); arrowhead indicates the scratch injury site.] Note the low level of Luman disappearance from the axon tip proximal to the injury when NLS peptide is added. (*B*) Dual-tagged Ad/GFP-Lu-RFP was intrathecally injected at the L5 DRG level and left for 7 d before a unilateral 48-h crush injury of the sciatic nerve. Nerve sections show nerve segments 1 mm proximal to the injury site, with the retrograde transport direction indicated by black arrows. Sections of intact sciatic nerves and corresponding L5 DRGs were prepared. The localization of GFP (fused to the N terminus of Luman) and RFP (fused to the C terminus of Luman) was analyzed. GFP and RFP are colocalized in the uninjured nerves, and are not observed in the uninjured DRG neuronal nuclei. [Scale bars, 100 μm (nerve) and 50 μm (DRG).].

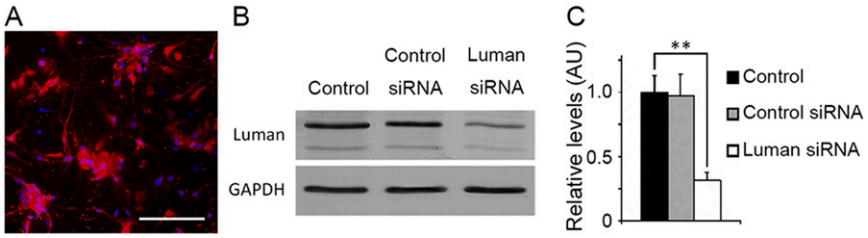


Fig. S6. Luman siRNA effectively knocks down Luman expression in 24-h injury-conditioned DRG neurons cultured for 48 h. (*A*) Transfection efficiency test of siRNA. Dissociated DRG neurons were transfected at a final concentration of 10 nM with the TYE 563 DS Transfection Control duplex and imaged 24 h posttransfection (red, labeled siRNA; blue, nuclei). Most cells can be transfected with labeled siRNA. [Scale bar, 200 μm.] (*B*) Expression level of Luman as analyzed by Western blot from protein samples of cultured 24-h injury-conditioned DRG neurons transfected with control nontargeting siRNA (control siRNA) or rat Luman-specific siRNA (Luman siRNA) 48 h previously. A representative blot is shown. (*C*) Quantification of relative changes in Luman levels detected in Western blots from three separate experiments as in *B*. Note: Luman siRNA significantly decreases the level of Luman detected as normalized to control injury-conditioned neurons (arbitrary units; mean ± SEM; $^{**}P < 0.01$).

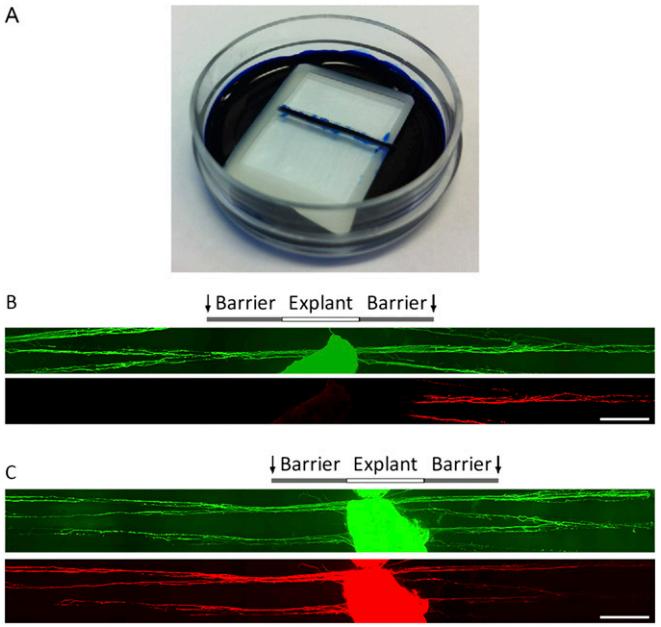
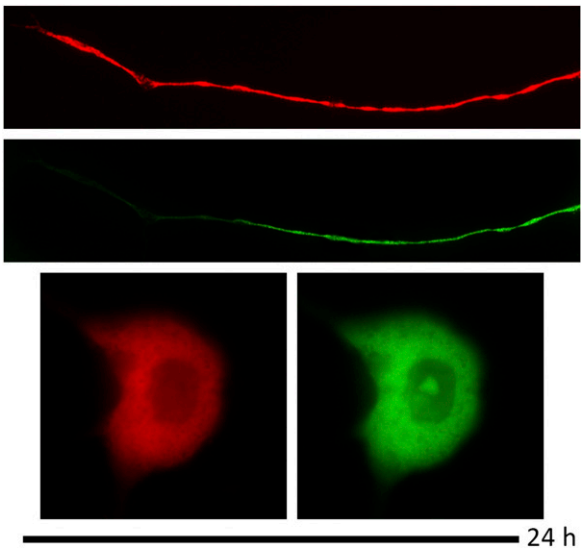


Fig. S7. Axon-specific Luman knockdown. (*A*) Compartmented culture model demonstrating compartmentally restricted properties by lack of Brilliant Blue diffusion over 24 h. (*B*) Representative fluorescence photomicrograph montages of axonal outgrowth detected with β-III-tubulin immunofluorescence (green) from 24-h injury-conditioned L4,5,6 DRG mini explants. Explant compartment and silicon barrier regions (gray) are indicated. Right axon compartments were transfected with TYE 563 DS Transfection Control duplex (red). Note that the fluorescently labeled siRNA transfection control (red) remains localized to the compartment in which it was delivered. (Scale bar, 1 mm.) (*C*) Representative fluorescence photomicrograph montages of axonal outgrowth detected with β-III-tubulin immunofluorescence (green) and Luman immunofluorescence (red) from L4,5,6 DRG mini explants. Explant compartment and silicon barrier regions (gray) are indicated. Left axon compartments were transfected with negative control nontargeting siRNA, whereas right axon compartments were transfected with Luman siRNA. (Scale bar, 1 mm.) Note that Luman protein expression is only knocked down in the compartment that the Luman siRNA was delivered into (right).



Movie S1. Movie showing retrograde transport of GFP-tagged Luman N terminus. Naïve DRG neurons cultured on coverslips were transduced with dual-tagged Ad/GFP-Lu-RFP for 48 h before receiving an in vitro scratch injury. GFP and RFP signals were then recorded for 24 h with a representative axon proximal to the injury, and the corresponding cell body is shown. Over the 24-h postinjury period, GFP signal in the axon proximal to the injury decreased in a retrograde manner toward the cell body, whereas GFP in the cell body and nucleus increased. RFP signal appeared unchanged.

Movie S1