hnRNPs Interacting with mRNA Localization Motifs Define Axonal RNA Regulons*

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mRNA translation in axons enables neurons to introduce new proteins at sites distant from their cell body. mRNA-protein interactions drive this post-transcriptional regulation, yet knowledge of RNA binding proteins (RBP) in axons is limited. Here we used proteomics to identify RBPs interacting with the axonal localizing motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs, revealing many novel RBPs in axons. Interestingly, no RBP is shared between all four RNA motifs, suggesting graded and overlapping specificities of RBP-mRNA pairings. A systematic assessment of axonal mRNAs interacting with hnRNPs H1, hnRNK, and hnRNPs F, and hnRNPs K, proteins that bound with high specificity to Nrn1 and Hmgb1, revealed that axonal mRNAs segregate into axon growth-associated RNA regulons based on hnRNPs interactions. Axotomy increases axonal transport of hnRNPs H1, F, and K, depletion of these hnRNPs decreases axon growth and reduces axonal mRNA levels and axonal protein synthesis. Thus, subcellular hnRNPs-interacting RNA regulons support neuronal growth and regeneration.

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mRNAs are actively transported into axons where they are used to synthesize new proteins (1). These locally generated proteins contribute to growth and function of axons as well as retrograde signaling for injury responses and survival in the peripheral nervous system (PNS) (2, 3). The transport of mRNAs into axons and their translation within axons can be regulated by extracellular stimuli (4). RNA binding proteins (RBPs) interacting with structural motifs within these mRNAs drive these post-transcriptional mechanisms (5). For example, zip-code binding protein 1 (ZBP1, also called IGF-II mRNA binding (IMP1)) protein was shown to bind to a 56 nucleotide (nt) stem-loop structure in the 3’ untranslated region (UTR) of β-actin mRNA (Actb), and this binding is necessary for axonal transport of the mRNA (6, 7). Other RBPs implicated in axonal mRNA transport include nucleolin (Ncl), HuD (also called ELAVL4), hnRNPs Q, hnRNPs R, splicing factor proline and glutamine-rich (SFPQ), fragile X mental retardation (FMRP), Hermes, TRF2-S, and TDP-43 proteins (8–17). Despite increased insight into RNA transport and translation, including the identification of literally thousands of axonal mRNAs (18, 19), relatively few RBPs have been identified in axons.

In other cellular systems, mRNAs encoding proteins with complementary functions have been shown to share RBPs that regulate their stability (20). This led to the notion of “RNA regulons,” where cohorts of mRNAs encoding proteins with complementary functions are co-regulated by shared RBPs. However, it is not known if the RNA regulon concept applies to subcellular compartments. Here, we have used axonal RNA localization motifs as “bait” coupled with mass spectrometry (MS) to identify the RBPs that bind to Nrn1 (also called Cortical Plasticity Gene 15), Hmgb1 (also called Amphoterin), Actb, and Gap43 mRNA localization motifs. Many interacting proteins were uncovered for each motif by this RNA affinity MS (RAMS) approach, but we found no protein shared by all four motifs. This suggests that pairing combinations of RBPs with localization motifs provides a level of specificity for axonal mRNA cohorts. RNA co-immunoprecipitation (RIP) with axonal heterogeneous nuclear ribonucleoproteins (hnRNPs) H1, F, and K, which showed high specificity binding to the Nrn1 and Hmgb1 mRNA motifs, segregates the axonal mRNA populations into binding cohorts, thus defining axon growth-associated RNA regulons based on hnRNPs interactions. Consistent with this link to axon growth, axotomy increases axonal transport of hnRNPs H1, F, and K, and depletion of these hnRNPs decreases axon growth and axonal protein synthesis.

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EXPERIMENTAL PROCEDURES

Animal Use and Neuron Cultures—All animal experiments were approved by institutional IACUC. 150–175 g adult male Sprague-Dawley rats were used for all experiments. Sciatic nerve crush injury and DRG cultures were performed as described (21). Dissociated L4–6 DRGs were cultured on poly-L-lysine (Sigma, St. Louis, MO) plus laminin (Millipore) coated coverslips or polyethylene-tetrahalate (PET) membrane inserts (1 μm pores; Falcon, Corning, NY). Axons were isolated from DRGs cultured on PET membranes as described (22).

For transfection, dissociated ganglia were pelleted at 100 × g for 5 min and resuspended in 20 μl nucleofector solution (Basic Neonur SCN Nucleofector kit; Lonza, Walkersville, MD). 2–3 μg of plasmid was electroporated using the Amazx Nucleofector™ II device (Lonza).

For siRNA transfections, 100 nM siRNA pools (Dharmacon, Lafayette, CO) were transfected using DharmaFECT 3 reagent (Dharmacon) at 3 h after plating DRGs. The siRNA sequences used in this study are shown in Supplemental Table S1. Non-targeting siRNAs (siCon; Dharmacon) were used as control. In some experiments, DRGs were transfected a second time with 100 nM siRNAs at 4 d in vitro (DIV), and then replated on DIV 6 as described (23).

Isolation of Sciatic Nerve Axoplasm—Axoplasm was obtained from rat sciatic nerve by extrusion in nuclear transport buffer (20 mM HEPES (pH 7.3); 110 mM potassium acetate, 5 mM magnesium acetate, 5 mM magnesium acetate) supplemented with protease/phosphatase inhibitor mixture (Roche, Basel, Switzerland) and RNasin Plus (Promega, Madison, WI) as described (24). Axoplasm preparations were cleared by centrifugation at 20,000 × g, 4 °C for 15 min and then processed for RNA affinity binding, immunoblotting, or immunoprecipitations as below. For crushed nerves, axoplasm was harvested proximal to the injury site. For naïve nerves, axoplasm was harvested from equal length of nerve taken from approximately the same anatomic location as the crushed nerves.

DNA Constructs—Myristoylated EGFP (GFPMYR) was used to screen for axonal localization by UTRs. GFPMYR-5′-ntrna1/3′-γ-actin construct has been previously described (25). The 1–95 and 95–188 nt segments of Nrn1 were generated by PCR and inserted restriction enzyme sites (Affi/BamHI) were used to replace the full length 5′UTR in GFPMYR-5′-ntrna1/3′-γ-actin construct. Bidirectional sequencing validated all amplified cDNA inserts.

Fluoresence In Situ Hybridization (FISH) and Immunofluorescence—For FISH/IF, transfected DRG neurons were fixed for 15 min in 4% paraformaldehyde (PFA). Digoxigenin (DIG) labeled GFP anti-sense cRNA probes were used to detect GFP as described; DIG labeled sense probes were used for control (26). Immunofluorescence for neurofilament (NF) was used to visualize neurons.

Immunofluorescence was performed as described (26). 4% PFA was used for fixation of all antibodies, except for anti-hnRNP K where ice-cold methanol was used. Primary antibodies consisted of rabbit anti-hnRNP H1 (Abcam, Cambridge, MA, 1:200), mouse anti-hnRNP K (Abcam, 1:200), rabbit anti-PurA (Abcam, 1:200) and a mixture of chicken anti-NF H, -NF M and -NF L (Aves labs, Tigard, OR, 1:500). FITC-conjugated donkey anti-rabbit, anti-mouse and Cy5-conjugated donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA, 1:200) were used as secondary antibodies.

Coverslips were mounted with Prolong Gold (Invitrogen, Wal-tham, MA). Fluorescent signals were captured with Leica DMi6000 epifluorescence microscope (Buffalo Grove, IL) and ORCA Flash ER CCD camera (Hamamatsu, Shizuoka Prefecture, Japan) using matched acquisition parameters (exposure time and gain) and any post-processing.

RNA Affinity Chromatography—RNA-protein pull-down for the RAMS procedure was performed as described (27). Briefly, biotin-conjugated RNA oligonucleotides (TriLink, San Diego, CA) were coupled to Streptavidin Dynabeads (SA; Invitrogen). After clearing, axoplasm from 7 d injury-conditioned rat sciatic nerve was incubated with oligonucleotide-bound beads for 4 h at 4 °C. Beads were precipitated using a magnetic rack and then washed extensively with 10 mM HEPES (pH 7.4), 3 mM magnesium chloride, 250 mM sodium chloride, 1 mM DTT and 5% glycerol. Bound proteins were eluted with 50 μg/ml RNase A (Sigma). Denatured proteins were fractionated by SDS/PAGE and then stained with Sypro Ruby solution; gel lanes were excised for MS.

In-gel Digestion and Mass Spectrometry—Protein bands were digested in-gel with trypsin as described (28). The extracted digests were separated in a NanoAcquity™ Ultraperformance UPLC system (Waters, Milford, MA) with incorporated nanoemitter (Thermo Scientific, Waltham, MA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. After equilibration of the column in 2% solvent B, an aliquot of each digest (5 μl) was injected, then the organic content of the mobile phase was increased linearly to 27% over 27 min, and then to 50% in 2 min. The liquid chromatography eluate was coupled to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific). Peptides were analyzed in positive ion mode and in information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in profile mode using the Orbitrap analyzer in the m/z range between 350 and 1400.

Peaklists were generated using PAVA in-house software (29), based on the RawExtract Script from Xcalibur v2.4 (Thermo Scientific). The peak lists were searched against the rat + human subset of the UniProt database as of June 17, 2013 (167,793 entries), using in-house ProteinProspector version 5.10.10 (http://prospector.ucsf.edu/prospector/mshome.htm). A randomized version of all en
tries was concatenated to the database for estimation of false discovery rates in the searches. Protein hits were considered significant when at least two peptide sequences matched a protein entry and the Prospector score was above the significance level. For identifications based on a single peptide sequence with high scores, the MS/MS spectrum was reinterpreted manually by matching all the observed fragment ions to a theoretical fragmentation obtained using MS Product (Protein Prospector) (30). supplemental Table S7 details protein identifications from single peptides with links for annotated spectra.

QSpec was used to determine the fold change (FC) between spectral counts of peptides bound to control or target RNA and the false discovery rates (FDR) (31). Log2FC cut-offs were 0.7 and −0.7 and FDR cut-off was 0.05. Normalized enrichment index compared with spectral counts normalized for protein molecular weight was used to incorporate binding to binding control. Equation for calculating enrichment index is shown in in Supplemental Table S2A.

RNA Immunoprecipitation (RIP)-Sequencing—Preclearing, primary antibody incubations, and precipitations were performed at 4 °C with rotation. Axoplasm isolates were precleared with Protein A-Dynabeads (Invitrogen) for 30 min, followed by incubation with primary antibodies for 3 h and then 2 h with Protein G-Dynabeads (Invitrogen). 5 μg each of the following primary antibodies were used: mouse anti-FLAG (Sigma), rabbit anti-hnRNP H1 (Abcam), mouse anti-hnRNP F (Thermo Scientific), mouse anti-hnRNP K (Abcam) and mouse anti-La/SSB (BD Biosciences, San Jose, CA) antibodies. Bound RNA was purified as above. For analyses by reverse transcription-coupled droplet digital PCR (RT-ddPCR), SsoAdvanced PreAmp kit (Bio-Rad, Hercules, CA) was used to pre-amplify cDNA for ddPCR per the manufacturer’s protocol.

RNA-sequencing libraries were prepared using the NuGEN Ovation RNA Ultra Low Input kit (500 pg, San Carlos, CA) and TruSeq Nano (Illumina, San Diego, CA). Libraries were indexed and sequenced over 3 lanes by HiSeq4000 (Illumina) with 69 bpb paired-end reads. Quality control (QC) was performed on base qualities and nucleotide composition of sequences, to identify problems in library preparation or sequencing. Reads were trimmed and filtered after the QC before input to the alignment stage. Reads were aligned to the latest rat Rn6 reference genome using the STAR spliced read aligner (ver 2.4.0). Average input read counts were 61.7 M and average percentage of uniquely aligned reads was 56%. Total counts of read-fragments aligned to known gene regions within the rat (rn6) Ensembl reference annotation were used as the basis for quantification of gene expression. Fragment counts were derived using HTSeq program (ver 0.6.0). RNAs with at least 10 counts in at least one condition across all replicates were retained for differential expression analysis, which was performed using EdgeR (ver 3.14.0). Average input read counts were 61.7M and average percentage of input to the alignment stage. Reads were aligned to the latest rat Rn6 reference genome using the STAR spliced read aligner (ver 2.4.0). Average input read counts were 61.7 M and average percentage of uniquely aligned reads was 56%. Total counts of read-fragments aligned to known gene regions within the rat (rn6) Ensembl reference annotation were used as the basis for quantification of gene expression. Fragment counts were derived using HTSeq program (ver 0.6.0). RNAs with at least 10 counts in at least one condition across all replicates were retained for differential expression analysis, which was performed using EdgeR (ver 3.14.0).

Relative mRNA enrichment in the RNA-seq data sets for each RBP compared with both input and anti-FLAG RIP RNA using the online RRHO server (http://systems.ucmp.ucla.edu/rankrank/index.php) with step size set at 100 (32). Scripts used in the RNA sequencing analyses are available at https://github.com/icrn/RNAseq-PIPELINE.git. Raw and processed data were deposited within the Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo, accession number: GSE103444).

Filtering RIP-seq Data Using Sensory Neuron-specific Translatomes—Sensory neuron specific translatome data were taken from a recent study that used RiboTag mice crossed with different Cre lines (Adv, Islt, Runx3, and Dhh) for cell specific expression (33). These translatome data were used to filter the axoplasm interactome lists for each RBP. For this, the RiboTag (mouse) and RIP-seq (rat) data were mapped to generate Ensembl gene names for each mRNA. dbOrtho (https://biodbnet-abcc.ncifcrf.gov/dbInfo/examples.php#dbOrtho) was then used to determine identities of overlapping mRNAs between the mouse and rat data sets. Rat orthologs were identified for more than 80% of the mouse genes from the neuron-specific translatome data set.

Motif Discovery in RBPs Interactome—Binding motifs enriched in the RBP-interacting mRNAs were analyzed by Homer motif discovery software (findMotifs.pl) (ver 4.9) (http://homer.ucsd.edu/homer/motif/index.html). We used the mouse refSeq with reported full-length mRNAs (5′UTR, CDS and 3′UTR) as source for motif discovery.

GO and IPA Analysis—Proteins identified by RAMS as interacting with different RNA oligonucleotides were examined for enrichment of GO terms using GOrilla (http://cbl-gorilla.cs.technion.ac.il/) (34). Target protein list and background list, which included all the proteins identified by MS, were submitted to identify enriched categories. The pathway enrichment analysis of the gene clusters was conducted with the R package clusterProfiler (35). The Bioconductor annotation packages GO.db, KEGG.db, and org.Hs.eg.db were used as references for the enrichment analysis.

Axonal mRNAs from RIP-Seq were examined for functional annotation by GO and IPA analysis. GO enrichment analysis was performed by submitting the list of mRNAs interacting with a hnRNP along with the background list to GOrilla. RBP-interacting mRNAs were mapped to canonical signaling pathways in the Ingenuity Pathways Knowledge Base using the entire list of mRNAs obtained by sequencing axoplasmic RNA as a background. One tailed Fisher’s exact test was used to calculate the p value for nonrandom association of mRNA ensembles with a given pathway.

Subcellular Protein Fractionation—Dissociated DRG neurons were plated on porous membranes (3 μm pore diameter) and cultured for 3 d. After rinsing with PBS three times, lower membrane surfaces were scraped to collect the axonal fraction that was then lysed in 1× Laemmli buffer. The upper membrane surface was then harvested in 20 mM HEPES (pH 7.4), 10 mM KCl, 1 mM MgCl2, 0.2% Triton X-100 and 1× protease inhibitor mixture (extraction buffer). Cells were lysed by gentle pipetting and then centrifuged at 15,000 × g for 20 min to pellet nuclei. Supernatant (cytoplasmic fraction) was denatured by addition of 4× Laemmli buffer. The pellet was washed three times with extraction buffer and then lysed with 1× Laemmli buffer as the nuclear fraction.

Immunoblotting—Protein lysates were quantified for yield by Bradford assay or BCA assay. Denatured protein lysates or aliquots from RIPs were fractionated by SDS-PAGE and transferred to PVDF or nitrocellulose membranes. For lysates, equivalent protein mass was used; for RIP analyses, protein yields were normalized prior to RIP, and then equivalent fractions of the RIPs were analyzed. After transfer, membranes were blocked and probed with primary and HRP-conjugated secondary antibodies as described (36). The following primary antibodies were used: rabbit anti-hnRNP H1 (Abcam, 1:5000), mouse anti-hnRNP F (Thermo Scientific, 1:1000), mouse anti-hnRNP K (Abcam, 1:2000), rabbit anti-H1/F (Abcam, 1:5000), rabbit anti-hnRNP K (Abcam, 1:2000), rabbit anti-Elav1/HuR (Cell Signaling, Danvers, MA, 1:1000), rabbit anti-Nucleolin (Abcam, 1:1000), rabbit anti-5-Hydroxytryptamine Receptor 2 (Abcam, 1:1000), rabbit anti-Histone H3 (Sigma, St. Louis, MO, 1:1000), mouse anti-α Tubulin (Cell Signaling, 1:5000), mouse anti-β Tubulin (Cell Signaling, 1:5000), mouse anti-β Actin (Cell Signaling, 1:5000), mouse anti-EF1α (Cell Signaling, 1:5000), mouse anti-GAPDH (Abcam, 1:2000), mouse anti-Lamin A/C (Santa Cruz Biotechnology, San Diego, CA, 1:3000), and rabbit anti-α Tubulin (Cell Signaling, 1:5000). HRP-conjugated anti-mouse or -rabbit antibodies (Cell Signaling, 1:2000) were used for secondary antibodies. For detection of immunoprecipitated hnRNP proteins, anti-mouse and anti-rabbit Trueblot kit (Rockland, Pottstown, PA) was used to minimize detection of denatured IgGs. Immune complexes were detected with ECL PrimeTM (GE Healthcare, Pittsburgh, PA).

RNA Isolation and PCR Analyses—RNA was isolated from dissociated DRG cultures using the RNeasy Microisolation kit and from whole DRGs using Trizol (Invitrogen). Fluorimetry with Ribogreen (Invitrogen) was used for RNA quantification. RNA samples were reverse...
neurons that were transfected with GFPMYR plus nt 1–188, 1–94 or 95–188 of rat GFP mRNA and immunofluorescent images for neurofilament (NF) protein in cell bodies and corresponding distal axons of cultured DRG are shown as mean pixel intensity

Briefly, neurons were incubated with 20
thesis Assay Kit

Nrn1 and had previously shown that intra-axonal translation of proteins in cultured DRG neurons, lyzed by anti-NF were acquired by tile-scanning of entire coverslips and ana-

Taqman (Integrated DNA Tech, Skokie, IL) detection. transcribed and used for ddPCR with either Evagreen (Bio-Rad) or Taqman (Integrated DNA Tech, Skokie, IL) detection.

Nascent Protein Synthesis Assay—To visualize newly synthesized proteins in cultured DRG neurons, Click-IT™ Plus OPP Protein Synthesis Assay Kit (Invitrogen) was used per manufacturer’s instruction. Briefly, neurons were incubated with 20 μM O-propargyl-puromycin (OPP) for 30 min at 37 °C. OPP-labeled proteins were detected by crosslinking with Alexa Fluor-594 picolyl azide. Cells were fixed, and coverslips were mounted with Prolong Gold Antifade mounting solution (Invitrogen) and imaged with Leica DMi6000 epifluorescent microscope as above. Morphology from DIC imaging was used to visualize neuronal processes and cell bodies, and to distinguish neurons from glial cells. ImageJ was used to quantify the Puromycin signals in cell bodies and distal axons.

Experimental Design and Statistical Rationale—All experiments were performed in at least three biological replicates and are reported as mean ± standard deviation (S.D.) or standard error of the mean (S.E.) as indicated. Student’s t test or one-way ANOVA with pairwise comparisons and Tukey post-hoc was used to determine significance differences between groups. Prism software (GraphPad, San Diego, CA) was used for all statistical analyses.

RESULTS

Defining the Axonal Localization Motif of Nrn1 mRNA—We had previously shown that intra-axonal translation of Hmgb1 and Nrn1 supports axon growth (25, 36). Axonal localization of Hmgb1 mRNA is driven through a 60 nt motif in its 3′ UTR, and the Hmgb1 mRNA is constitutively transported into sensory axons (36). Nrn1 mRNA’s transport into axons is increased during regeneration and its 5′ UTR drives its localization (25). We reasoned that the differential transport of these two mRNAs could be leveraged to test for possible occurrence of axonal RNA regulons, hence we sought to narrow down the 188 nt Nrn1 mRNA 5′ UTR to a shorter RNA segment suitable for RAMS (27). For this, we transfected rat primary dorsal root ganglion (DRG) neurons with a reporter mRNA containing varying portions of Nrn1 mRNA 5′ UTR (NCBI accession # NM_053346.1), the coding sequence of myristoylated GFP (GFPMYR), and the non-localizing 3′ UTR of γ-actin mRNA (Actg). As previously shown (25), GFP mRNA localized into axons when it included Nrn1 mRNA nt 1–188. In contrast, GFPMYR with Nrn1 mRNA nt 1–94 showed axonal GFP mRNA signals indistinguishable from the sense probe (data not shown), whereas nt 95–188 revealed robust axonal GFP mRNA signals (Fig. 1A, 1C). Axonal localization of GFP mRNA was lost upon further deletions of nt 95–188 segment (data not shown), indicating that this 5′ UTR region is necessary and sufficient for its axonal localization. Nrn1 mRNA nt 95–188 shows higher sequence conservation across species than Nrn1 mRNA nt 1–94 (supplementary Fig. S1A). Although an RBP can show primary sequence specificity, in many cases RBPs recognize motifs in the context of secondary structures (38). Secondary structure predictions using aligned Nrn1 sequences show a stem-loop RNA structure for nt 95–188 compared with relatively unstructured nt 1–94 (supplemental Fig. S1B–S1C). These findings indicate that Nrn1 mRNA’s nt 95–188 may serve as a binding motif for axonal RBPs.

Distinct Axonal Protein Populations Bind to the Localizing Motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs—We used RAMS with biotinylated RNA oligonucleotides to identify RBPs in sciatic nerve axoplasm that bind the localizing motifs of Nrn1 and Hmgb1 mRNAs. RNAs corresponding to rat Nrn1

Fig. 1. nt 95–188 in Nrn1’s 5′ UTR are sufficient for its axonal localization. A, Representative fluorescent in situ hybridization images for GFP mRNA and immunofluorescent images for neurofilament (NF) protein in cell bodies and corresponding distal axons of cultured DRG neurons that were transfected with GFPMYR plus nt 1–188, 1–94 or 95–188 of rat Nrn1 (5′ Nrn11–188, 5′ Nrn11–94, and 5′ Nrn195–188, respectively) are shown (Scale bars = 20 μm). B–C, Quantification of RNA signals in the cell body (B) and distal axons (C) from replicate cultures as in A are shown as mean pixel intensity ± S.E. (N ≥ 45 neurons over 3 separate culture/transfections; ***, p < 0.001 by one-way ANOVA with pair-wise comparison and Tukey post-hoc).
nt 95–188 and Hmgb1 nt 706–766 (NM_012963.2) were used as “target RNAs.” Nrn1 nt 1–94 and Hmgb1 nt 2170–2230, a nonlocalizing GC-matched 3’UTR segment, were used as “control RNAs.” A second control for each RAMS consisted of biotin-saturated SA beads (“biotin control”) (27). SDS/PAGE showed many bound proteins under all three conditions, but target RNA binding was clearly different from control RNA and biotin control (supplemental Fig. S1D).

MS analyses showed many proteins bound to localizing motifs of Nrn1 and Hmgb1 mRNAs. Replicate RAMS assays were normalized for protein yields across the individual pull-downs for target RNAs, control RNAs, and biotin controls (supplemental Table S2B–S2C). We tested for protein enrichment in the Nrn1 nt 95–188 and Hmgb1 nt 706–766 pull-downs by two different analytic approaches. First, we compared relative enrichment of target versus control RNA using QSpec, which tests for differential expression in spectral count data using a generalized linear mixed effects model and hierarchical Bayes estimation (31). QSpec analysis showed several proteins enriched in the Nrn1 nt 95–188 and Hmgb1 nt 706–766 pull-downs compared with their control RNAs (Fig. 2A–2B, supplemental Table S3A–S3B). Second, we incorporated the biotin control interactions into consideration by calculating an “RNA target enrichment index” (supplemental Table S2A) and comparing this to spectral counts normalized for molecular weight to account for relative protein yields. Several proteins showed enriched binding to Nrn1 nt 95–188 and Hmgb1 nt 706–766 compared with controls (Fig. 2C–2D). Proteins with significant enrichment for Nrn1 nt 95–188 and Hmgb1 nt 706–766 binding were largely consistent between the two analyses, and the “high specificity interactors” for these localization motifs notably include several known RBPs (Fig. 2A–2D).

As only a few high specificity interactors overlapped between Nrn1 nt 95–188 and Hmgb1 nt 706–766, we expanded the data set to test for RBPs binding to Gap43’s 3’UTR AU-rich element (ARE; nt 1211–1250, NM_017195.3) and Actb’s 3’UTR localization motif (nt 1206–1261, NM_031144.3) (8). The axonal localization and translational regulation of Gap43 and Actb mRNAs are distinct from those of Nrn1 and Hmgb1 (8, 39), so we reasoned these mRNAs would provide a rigorous test for potential RBP sharing between motifs. Because known RBPs also purified with the control Nrn1 and Hmgb1 RNA sequences, we used a scrambled oligonucleotide as control mRNA in the Gap43 and Actb pull downs to determine if those RBPs represented nonspecific RNA interactions. High specificity interacting proteins were seen in RAMS for both Gap43 and Actb mRNA motifs (supplemental Fig. S2C–S2H and supplemental Tables S2D–S2E, S3C–S3D). Some RBPs were shared with the Nrn1 and Hmgb1 mRNA localization motifs, however no high specificity interacting protein was shared by the Nrn1, Hmgb1, Gap43, and Actb mRNAs motifs (Fig. 2F, Table I).

Gene Ontology (GO) analyses for both summed high specificity interactors for Nrn1, Hmgb1, Gap43, and Actb motifs and for Nrn1 nt 1–94, Hmgb1 nt 2170–2230 and scrambled RNA showed a preponderance of nucleic acid binding terms, including RNA binding and splicing (supplemental Fig. S3). Even though these GO terms are clearly distinct from those of biotin, there were only minor differences between GO terms highlighted for axon localizing RNA motifs and control RNAs (supplemental Fig. S3). This is perhaps not a surprising finding given RNA was the bait for both purifications. Nonetheless, the GO analyses show greater enrichment for RNA interacting categories for Nrn1 and Hmgb1 control RNAs than for the scrambled RNA probe (supplemental Fig. S3).

Axonal hnRNPs Interact with Nrn1 and Hmgb1 mRNAs—Immunoblotting was used to validate the RNA affinity pull-down for Nrn1 nt 95–188 and Hmgb1 nt 706–766 high specificity interactors. Higher levels of hnRNP H1, hnRNP F, hnRNP A3, PurA, and PurB were pulled down with Nrn1 nt 95–188 than with Nrn1 nt 1–94, and higher levels of hnRNP K, ELAVL1, PurA, and PurB were pulled down with Hmgb1 nt 706–766 than with Hmgb1 nt 2170–2230. Further in accordance with RAMS data, higher levels of hnRNP K, ELAVL1, and Ncl were pulled down with Nrn1 nt 1–94 than with Nrn1 nt 95–188 (Fig. 2E). Notably, hnRNP H1 and F showed higher binding to Hmgb1 nt 706–766 than to Hmgb1 nt 2170–2230, and neither of these proteins were identified as Hmgb1 nt 706–766 high specificity interactors (Fig. 2B, 2E). However, calculating enrichment indices from raw spectral counts showed enrichment indices of 0.62 ± 0.09 and 0.63 ± 0.05 for hnRNP H1 and hnRNP F, respectively (normalized enrichment indices were hnRNP H1 = 0.42 ± 0.12 and hnRNP F = 0.43 ± 0.11). Thus, our normalization procedure for the RAMS analyses is stringent to a degree that a few real interactors may be discarded, paying the price of a few false negatives for robustness of the final prioritized candidates. Indeed, every protein that was identified as a high specificity interactor for Nrn1 nt 95–188 and Hmgb1 nt 706–766 was validated by immunoblotting (Fig. 2E), except for hnRNP AB and Kas, for which suitably specific antibodies are not available.

The studies above show that several RBPs localize to peripheral nerve axons and some interact with the localizing motifs in Nrn1’s 5’UTR and Hmgb1’s 3’UTR at high specificity. We then asked if the endogenous Nrn1 and Hmgb1 mRNAs might bind to these axonal RBPs. Antibodies to hnRNP H1, F, and K proved suitable for immunoprecipitation (IP) from sciatic nerve axoplasm (Fig. 3A). Reverse transcription-coupled droplet digital PCR (RTdPCR) revealed that Nrn1 and Hmgb1 mRNAs were enriched in the hnRNP K IPs compared with control. Both mRNAs were detected in the hnRNP H1 IPs, but only Nrn1 mRNA was significantly enriched (Fig. 3B–3C). Unfortunately, lower RNA quantities were isolated from hnRNP F IPs and no significant enrichment of Nrn1 or
Identification of proteins interacting with axon localizing RNA motifs. A–B, Volcano plots from QSpec analyses of RAMS data for axonal mRNA motifs versus control RNAs of Nrn1 (A) and Hmgb1 (B) are shown. Only protein hits with false-discovery rates (FDR) ≤ 0.05 over three biological replicates are included. Thresholds of 0.7 and −0.7 for log2FC (blue vertical lines) and 0.05 for p value (red horizontal line) are shown. Candidate proteins with enriched binding to localizing motifs are in blue font, and those with enriched binding to control RNAs are in gray font. See supplemental Tables S2 and S3 for spectral count data and analyses.

C–D, Target RNA enrichment indices from RAMS data for Nrn1 nt 95–188 versus Nrn1 nt 1–95 plus biotin-saturated beads (C) and Hmgb1 nt 706–766 versus Hmgb1 nt 2,170–2,230 plus biotin-saturated beads (D) are shown relative to the protein spectral counts normalized to molecular weight. The black hatched lines indicate median values and red hatched lines indicate 99% confidence intervals (CI). Proteins showing high specificity interactions with target RNA are in blue font, and those showing higher interactions with control RNA and beads are in gray font. Refer to and supplemental Fig. S2A–S2B for analyses of Nrn1 1–94 and Hmgb1 2170–2230. E, Representative immunoblots for sciatic nerve axoplasm protein binding to biotin Nrn1 nt 1–94 and nt 95–188 (left) or Hmgb1 nt 706–766 and nt 2,170–2,230 (right) are shown. “Input” lane shows axoplasm isolate (5%). F, Venn diagram representing enriched proteins shared between QSpec and Enrichment Index analyses for axonal localizing motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs is shown. See also Table I and supplemental Fig. S2 and S3.

Fig. 2. Identification of proteins interacting with axon localizing RNA motifs. A–B, Volcano plots from QSpec analyses of RAMS data for axonal mRNA motifs versus control RNAs of Nrn1 (A) and Hmgb1 (B) are shown. Only protein hits with false-discovery rates (FDR) ≤ 0.05 over three biological replicates are included. Thresholds of 0.7 and −0.7 for log2FC (blue vertical lines) and 0.05 for p value (red horizontal line) are shown. Candidate proteins with enriched binding to localizing motifs are in blue font, and those with enriched binding to control RNAs are in gray font. See supplemental Tables S2 and S3 for spectral count data and analyses. C–D, Target RNA enrichment indices from RAMS data for Nrn1 nt 95–188 versus Nrn1 nt 1–95 plus biotin-saturated beads (C) and Hmgb1 nt 706–766 versus Hmgb1 nt 2,170–2,230 plus biotin-saturated beads (D) are shown relative to the protein spectral counts normalized to molecular weight. The black hatched lines indicate median values and red hatched lines indicate 99% confidence intervals (CI). Proteins showing high specificity interactions with target RNA are in blue font, and those showing higher interactions with control RNA and beads are in gray font. Refer to and supplemental Fig. S2A–S2B for analyses of Nrn1 1–94 and Hmgb1 2170–2230. E, Representative immunoblots for sciatic nerve axoplasm protein binding to biotin Nrn1 nt 1–94 and nt 95–188 (left) or Hmgb1 nt 706–766 and nt 2,170–2,230 (right) are shown. “Input” lane shows axoplasm isolate (5%). F, Venn diagram representing enriched proteins shared between QSpec and Enrichment Index analyses for axonal localizing motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs is shown. See also Table I and supplemental Fig. S2 and S3.
**Identification of Novel RNA Binding Proteins for Axons**

**TABLE I**

| Summary of proteins showing high specificity interaction with axonal localizing elements of Nrn1, Hmgb1, Gap43, and Actb mRNAs |
|---------------------------------------------------------------|
| **Nm1**  | **Hmgb1** | **Gap43** | **Actb** |
| hnRNP AB  | +         | +         | +        |
| PurA      | +         | +         | +        |
| PurB      | +         | +         | +        |
| hnRNP A3  | +         | +         | +        |
| hnRNP D   | +         | +         | +        |
| hnRNP F   | +         | +         | +        |
| hnRNP H1  | +         | +         | +        |
| Elav1 (HuR) | +     | +         | +        |
| Kars      | +         | +         | +        |
| hnRNP K   | +         | +         | +        |
| Ncl       | +         | +         | +        |
| PTBP1     | +         | +         | +        |
| hnRNP A2B1| +         | +         | +        |
| KHSRP     | +         | +         | +        |
| Fupb1     | +         | +         | +        |
| LOC684558 | +         | +         | +        |
| hnRNP A1  | +         | +         | +        |
| Mt2       | +         | +         | +        |
| Rhase1    | +         | +         | +        |
| hnRNP U   | +         | +         | +        |
| PCBP2     | +         | +         | +        |
| Ddx15     | +         | +         | +        |
| hnRNP L   | +         | +         | +        |
| PCBP3     | +         | +         | +        |
| hnRNP R   | +         | +         | +        |
| Ybx1      | +         | +         | +        |
| Dars      | +         | +         | +        |
| Ssb       | +         | +         | +        |
| Pabpc1    | +         | +         | +        |
| Dis3d2    | +         | +         | +        |
| Purf60    | +         | +         | +        |
| PTBP2     | +         | +         | +        |
| Srsf3     | +         | +         | +        |
| Ybx3      | +         | +         | +        |
| Hdlbp     | +         | +         | +        |
| PTBP3     | +         | +         | +        |
| Rtc1      | +         | +         | +        |
| Fupb3     | +         | +         | +        |
| Ashg      | +         | +         | +        |
| Paics     | +         | +         | +        |
| Eef2      | +         | +         | +        |
| hnRNP H2  | +         | +         | +        |
| Rpsa      | +         | +         | +        |
| Bub3      | +         | +         | +        |
| Tardbp    | +         | +         | +        |
| Col14a1   | +         | +         | +        |
| hnRNP A2B1| +         | +         | +        |
| Ddx1      | +         | +         | +        |
| hnRNP DL  | +         | +         | +        |
| Rtc3      | +         | +         | +        |
| Tia1      | +         | +         | +        |
| Rab5c     | +         | +         | +        |

*Hmgb1* mRNAs was seen in these IPs, perhaps because of lower efficiency of the anti-hnRNP F antibody (Fig. 3A). This co-IP of endogenous mRNAs showed that H1 and K can bind to *Nm1* mRNA, which is not surprising because the RAMS data indicated that hnRNP H1 interacts with *Nm1* nt 95–188 and hnRNP K interacts with *Nm1* nt 1–94 (Fig. 2A, 2E and supplemental Fig. S2A). To determine if the select interaction of hnRNP H1 and F with *Nm1* mRNA nt 95–188 is unique to the synthetic RNAs used for RAMS, we asked if a Flag-tagged *Nm1* mRNA would still co-IP with hnRNP H1 and K in a cellular context when nt 1–94 or 95–188 were deleted. For this, DRG cultures were transfected with the *Nm1* 5’UTR truncation constructs indicated in Fig. 3D and processed for immunoprecipitation with control, anti-hnRNP H1 or anti-hnRNP K antibodies. Consistent with the RAMS analyses, amplifying the *Flag*-tagged *Nm1* mRNAs from these IPs showed that hnRNP H1 bound significantly more *Nm1* mRNA nt 95–188 than nt 1–94, whereas hnRNP K bound significantly more *Nm1* mRNA nt 1–94 compared with nt 95–188 (Fig. 3E). These results support the RAMS findings that different hnRNP proteins associate with axonal mRNA through interaction with distinct RNA motifs.

*RNA Coimmunoprecipitation Analyses of Axonal hnRNP H1, hnRNP F, and hnRNP K Segregate Axonal mRNAs Into Growth-associated Cohorts*—Recent RIP approaches in 293T and HeLa cells focused on hnRNP F and H1 as coordinators of RNA splicing (40, 41). We reasoned that the axoplasmic preparations would allow us to uniquely address these RBPs’ cytoplasmic interactors, and specifically their axonal RNA interactors. In addition to hnRNP H1, hnRNP F, and hnRNP K, we analyzed RIP data for La/SSB, an RNA chaperone protein that also localizes to sciatic nerve axons (42). For each RIP, input RNAs were sequenced for mRNA levels and RIP-seq with anti-FLAG antibody was carried out to test for IP specificity/enrichment. Top mRNAs enriched in comparisons with both controls were identified using the *Rank-Rank Hypergeometric Overlap* (RRHO) algorithm (32) (Fig. 4A). All detected mRNAs were ranked for enrichment versus input RNA-seq and anti-FLAG antibody IP RNA-seq, and these ranks were compared using RRHO to identify a core set of mRNAs with high ranking for each RBP compared with these two control data sets. We identified 3588 enriched mRNAs for hnRNP H1, 3609 for hnRNP F, for 2557 for hnRNP K, and 1010 for La/SSB (Fig. 4B; supplemental Table S5). Comparison of these RBP–mRNA interactomes showed highest overlap between hnRNP H1 and F (2821 mRNAs; Fig. 4B), which is not unexpected based on previous studies of hnRNP H1 and F interacting mRNAs (40, 41).

To exclude nonneuronal RBP-RNA interactions from the RIP data sets, we used recently available cell-specific translatome profiles of DRGs to select neuronal-enriched mRNAs from the hnRNP H1, hnRNP F, and La/SSB interactomes. Rozenbaum et al. (2018) used RiboTag mice (43) crossed with different Cre lines specific to sensory neurons (Adv, Isl1, and Runx3) or Schwann/satellite cells (DHH) to generate HA tagged-ribosomal protein L22 (L22-HA) in a cell-specific manner (33). 6230 sensory neuron-enriched mRNAs were identified from these data by RRHO using neuronal (Adv, Isl1, and Runx3) or Schwann/satellite cells (DHH) to crossed with different Cre lines specific to sensory neurons (Adv, Isl1, and Runx3) or Schwann/satellite cells (DHH). We reasoned that the axoplasmic preparations would allow us to uniquely address these RBPs’ cytoplasmic interactors, and specifically their axonal RNA interactors. In addition to hnRNP H1, hnRNP F, and hnRNP K, we analyzed RIP data for La/SSB, an RNA chaperone protein that also localizes to sciatic nerve axons (42). For each RIP, input RNAs were sequenced for mRNA levels and RIP-seq with anti-FLAG antibody was carried out to test for IP specificity/enrichment. Top mRNAs enriched in comparisons with both controls were identified using the *Rank-Rank Hypergeometric Overlap* (RRHO) algorithm (32) (Fig. 4A). All detected mRNAs were ranked for enrichment versus input RNA-seq and anti-FLAG antibody IP RNA-seq, and these ranks were compared using RRHO to identify a core set of mRNAs with high ranking for each RBP compared with these two control data sets. We identified 3588 enriched mRNAs for hnRNP H1, 3609 for hnRNP F, for 2557 for hnRNP K, and 1010 for La/SSB (Fig. 4B; supplemental Table S5). Comparison of these RBP–mRNA interactomes showed highest overlap between hnRNP H1 and F (2821 mRNAs; Fig. 4B), which is not unexpected based on previous studies of hnRNP H1 and F interacting mRNAs (40, 41).

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mRNAs (Fig. 4C). With these data sets, overlap analyses showed that hnRNP H1 and F share 825 interacting mRNAs (62.5% of total hnRNP H1 and F-interacting mRNAs) and hnRNP H1, F, and K share 202 interacting mRNAs (12.1% of the total hnRNP H1, F, and K interacting mRNAs; Fig. 4D). Although enriched in the hnRNP H1, F, and K unfiltered RIP-seq data sets, Nrn1 and Hmgb1 mRNAs were filtered out of the neuron-enriched interactomes as these proteins derived from RRHO with DRG translatome data. This may reflect the fact that both Nrn1 and Hmgb1 are post-transcriptionally regulated after axotomy (25, 36) and the translatome data reflects translationaly active mRNAs from sensory neuron soma (33).

We took three approaches to test validity of these neuron-enrichment filtered RBP-mRNA interactomes. First, FPKM values of mRNAs belonging to each RBP-interactome were extracted from the RIP-seq data set and plotted relative to fold-change in input RNA. This showed a significant enrichment for the hnRNP H1-, F-, and K-specific mRNAs as well as those mRNAs shared between H1 and F (supplemental Fig. S4). Second, RTddPCR for 35 randomly selected hnRNP H1-, F-, and K-interacting mRNAs were directly tested for mRNA levels precipitating with RBPs from sciatic nerve axoplasm. mRNA copies for each RIP from RT-ddPCR analyses showed a significant linear correlation with FPKM values from the RIP-seq data (supplemental Fig. S5). The different y-intercepts for these analyses are because of different input levels for the RNA-seq and RTddPCR - the RNA-seq requires equivalent RNA mass, whereas the RTddPCR was performed based on equivalent fractions of the IPs. The weak correlation of La/SSB is likely because of the relatively low abundance of these mRNAs precipitated by La/SSB compared with other RBPs. Third, we performed de novo motif discovery on the neuron-enriched hnRNP H1, F, and K interactomes. The top 3 predicted hnRNP H1 and F motifs contain quite similar A/G-rich stretches, whereas motifs predicted for hnRNP K appeared distinct (Fig. 4E). A/G-rich motifs were also identified as direct binding motifs for hnRNP H1 in HEK 293T and HeLa cells (40, 41). Binding to Hmgb1 mRNA was only identified HEK 293T cells, and that was an intronic RNA interaction rather than to the mRNA (44); Nrn1 mRNA was not found in either cell line possibly because of low Nrn1 expression (40, 41).

GO term analysis showed several enriched terms and pathways shared between the hnRNP H1-, F-, and K-interacting mRNAs, with several related to axon growth mechanisms.
In contrast, these terms did not show as enriched in the GO terms for La/SSB interactome or mRNAs identified in the axoplasm used for the IPs (i.e. input; supplemental Fig. S6A–S6C). Furthermore, pathway analyses of the interactomes showed that “axon guidance” was more enriched in the hnRNP interactomes than the La/SSB inter-
Taken together, these data validate the RBP-mRNA interactomes developed from RRHO neuron-enrichment filtered hnRNP RIP analyses and emphasize the utility of this approach to selectively uncover RNA-protein interactions in peripheral axons.

Axonal hnRNP H1, F, and K Levels Increase with Axotomy—The GO analyses and IPA above suggest that axon growth-regulating RNA regulons are defined by hnRNP interactions. To test this possibility, we first asked if axonal levels of these proteins change during nerve regeneration. For this, we used immunoblotting to compare the relative levels of Nrn1 nt 95–188 or Hmgb1 nt 706–766 high specificity interacting proteins in sciatic nerve axoplasm after crush injury. Levels of hnRNP H1, F, and K were significantly increased in injured compared with naïve nerve (Fig. 5A, 5C). L4–5 DRG lysates did not show any detectable change in hnRNP H1, F, and K protein levels after nerve injury (Fig. 5A, 5B). There was also no change in levels of the mRNA encoding these proteins in injured L4–5 DRGs (data not shown), suggesting that the increased hnRNP levels in the regenerating axoplasm occur through post-translational mechanisms.

Because the studies above used extruded axoplasm, we asked if these RBPs could be visualized in distal axons. After an exhaustive trial of antibodies, fixation, and immunolabeling approaches, we identified a few RBP antibodies suitable for immunofluorescence. hnRNP H1, hnRNP K, and PurA were visualized in axons of cultured DRG neurons, and axonal signals for hnRNP H1 and K were more prominent in injury-conditioned neurons (Fig. 5D). The axonal RBP signals were punctate, like what has been reported for mRNAs in axons. Interestingly, the signals for hnRNP H1 and K appeared predominantly nuclear in Schwann cells, whereas the soma of the DRG neurons showed more prominent cytoplasmic signals (supplemental Fig. S7). By immunoblotting with lysates isolated from axonal, cytoplasmic and nuclear fractions of dissociated DRG cultures, levels of hnRNP H1, F and K proteins were significantly higher in nuclear compared with cytoplasmic fractions (Fig. 5E). However, axonal isolates showed that axonal fractions contained levels of hnRNP H1, F and K proteins comparable to the nuclear fractions (Fig. 5E). Considering that glial cells are also included in the nuclear and cytoplasmic fractions analyzed in these cultures, these data...
emphasize that these hnRNPs show robust localization into the axonal compartment.

hnRNP H1 and F Function to Support Axon Growth—Axonally synthesized Nrn1 and Hmgb1 proteins support axonal outgrowth from DRG neurons (25, 36). Thus, we asked if depletion of the high specificity RBP interactors for Nrn1 and Hmgb1 mRNAs might affect axon growth. Axon outgrowth from cultured DRG neurons was significantly decreased at 3 days in vitro (DIV) after treatment with hnRNP H1 and hnRNP F siRNAs. hnRNP K, A3, and AB siRNAs had no significant effect on axon growth, whereas PurA and PurB siRNAs caused a modest, but statistically significant decrease in axon length (Fig. 6). Although each of the target mRNAs was robustly depleted by the siRNAs (supplemental Fig. S8A), there was only modest reduction of hnRNP H1 and F proteins (immunoreactivity in soma relative to control: hnRNP H1 = 0.79 ± 0.04, p = 0.016 and hnRNP F = 0.81 ± 0.02; p = 0.002; data not shown). High stability of these RBPs might explain the mismatch between hnRNP H1 and F protein and mRNA levels with the siRNA transfections. To address this possibility, we used sequential siRNA transfections at DIV 0 and 4 that brought greater reduction of the proteins (supplemental Fig. S8B–S8D). There was more reduction in axon length for hnRNP H1- and F-depleted DRGs as well as decreased axon branching in hnRNP H1-, F-, and K-depleted DRGs (supplemental Fig. S8B–S8D). Together, these data point to axon growth functions for hnRNP H1, F, and K in adult sensory neurons, which is consistent with functional predictions derived from RAMS and subsequent RIP-seq analyses.

Combined Depletion of hnRNP H1, H2, and F Decreases Axonal mRNA Translation—Despite that axon growth was affected by siRNA-mediated decreases in hnRNP H1, F, and K, axonal levels of Nrn1 and Hmgb1 showed no change with these knockdowns (data not shown). hnRNP H1 is known to form a heterodimer with hnRNP F (45). hnRNP H2 has not been studied extensively but it is highly homologous to hnRNP H1, with greater 95% primary sequence identity. Thus, hnRNP H1, H2, and F could share functions, which is consistent with the overlapping mRNA interactomes of hnRNP H1 and F shown above. To test this possibility, we performed double and triple knockdown hnRNP H1 and H2 and hnRNP H1 + H2 + F (supplemental Fig. S8A). Single knockdown of hnRNP H2 had no significant effect on axon length or branching (Fig. 7A–7B). Combined knockdown of hnRNP
H1 + H2 and hnRNP H1 + H2 + F did not result in significant further reduction of axon length or branching compared with the single knockdowns of hnRNP H1 or F (Fig. 7A–7B). Despite no additive or synergistic effects with double and triple knockdowns, there was a significant decrease in axonal

Hmgb1 mRNA with hnRNP H1 + H2 + F siRNA transfections (Fig. 7C–7D). Axonal Nrn1 mRNA showed some decreases in both cell body and axons with these depletions but none reached statistical significance (supplemental Fig. S9B–S9C). Puromycylation assays also showed decreased translation
in axons and cell bodies after knockdown of hnRNP H1 + H2 and hnRNP H1 + H2 + F (Fig. 7E–7F). These data suggest overlapping functions of hnRNP H1, H2, and F for post-transcriptional regulation of gene expression in sensory neurons.

**DISCUSSION**

Axons of sensory, cortical, hippocampal, retinal ganglion, and motor neurons have been shown to contain complex mRNA populations by RNA-seq analyses of isolated axons (18, 19, 46–50). Despite identifying thousands of axonal mRNAs, exceptionally few RBPs have been found to date in axons. We used an unbiased proteomics approach to identify axonal RBPs that interact with the localizing motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs. These four axonal mRNAs are regulated by injury-induced axonal localization, injury-induced intra-axonal translation, neurotrophin-induced transport/translocation, and injury-induced transcription-coupled axonal localization, respectively (6, 8, 22, 25, 36). We observed a surprising diversity of RBPs showing high specificity binding to these mRNA localization motifs. Axonal localization was not previously known for most of the identified RBPs. No high specificity interacting proteins were shared between all four mRNA localization motifs, suggesting the existence of multiple mRNA-protein complexes defined by composition of RBPs interacting with these mRNA motifs. RIP-seq analyses for the RBPs further distinguished hnRNPs F, H1, and K as high specificity interactors for Nrn1 and Hmgb1 mRNAs. Axonal mRNAs encoding proteins linked to axon growth are enriched in the mRNA interactomes for hnRNP H1, F, and K compared with mRNAs enriched in the La/SSB interactome and those in the input axoplasm RNA-seq. Taken together, our data provide a unique and novel resource for axonal RBPs (Table S2). The axoplasm preparation method used here has previously been shown to be highly enriched in axonal proteins compared with non-neuronal proteins (63), indicating that the RAMS approach used here has uncovered RNA-interacting proteins from axons. The RBP–mRNA interactomes established here provide a cohort of bound axonal mRNAs that can be tested in the future for direct versus indirect RBP interactions. Nonetheless, there are limitations to the RAMS approach used here. First, both HuD and ZBP1 are known to bind to the localization motifs of Gap43 and Actb, but these proteins were not identified here (supplemental Table S2). The axoplasm preparation is enriched in axonal protein constituents but detergents are not used for isolation (24). Thus, RBPs that interact with cytoskeleton, such as HuD and ZBP1, would be missed in the RAMS. Second, the stringency we used for normalization of biological replicates unavoidably causes false negatives, with some relevant bound proteins failing significance criteria in the QSpec enrichment index analyses. hnRNP H and H1 binding to Hmgb1 nt 706–766 in the validation studies above are evidence of this. However, these stringent criteria increase confidence in the high specificity interactors identified by RAMS.

Axotomy is known to change neuronal gene expression programs, and this is in part by altering axonal transport of cargo proteins (64, 65). The increased levels that we found for hnRNP F, H1, and K in regenerating axons most likely occurs through post-transcriptional mechanisms (Fig. 6C–6D). Either increased transport into axons, local translation or increased stability within axons could account for these changes. Interestingly, the increased levels of axonal hnRNP F and H1 are in line with the increase in axonal Nrn1 transport after PNS nerve injury, and the increase in axonal hnRNP K corresponds to the
increase in Hmgb1 translation seen in regenerating axons (25, 36). Post-translational modifications have been shown to alter activity and/or subcellular localization of a few RBPs (6, 42, 66, 67). Thus, it will be of high interest to determine how more hnRNP F, H1 and K proteins localize into regenerating axons, particularly because knockdown of these proteins affects axon growth and combined knockdown of hnRNP H1, H2, and F decreased protein synthesis in axons.

In summary, we have identified RBPs that interact with localizing motifs of Nrn1, Hmgb1, Actb, and Gap43 mRNAs in regenerating sciatic nerve, substantially increasing the number of known axonal RBPs. The majority of the RBPs identified here were found to interact with only one or two of the four axonal mRNA localization motifs tested. MS analyses of proteins interacting with the RBPs Staufen and Barentsz in embryonic rat brain indicate at least two distinct dendritic RNP populations (60), and multiple dendritic RNPs have been defined by DEAD box protein content (68). Thus, the RBPs identified in the RAMS analyses here most likely constitute unique protein combinations for cohorts of axonally localizing mRNAs. This is supported by our RNA profiling of axonal hnRNP-bound mRNAs, which segregates axonal mRNAs into functional groups highly suggestive of subcellular RNA regulons.

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DATA AVAILABILITY

Data for protein identifications based on single peptides are available as individual annotated spectra at http://prospector.ucsf.edu/prospector/mshome.htm (see supplemental Table S7 for accession numbers). Raw and processed RNA co-immunoprecipitation sequencing data were deposited within the Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo, accession number: GSE103444). The raw MS proteomics data have been deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) with the data set identifier MSV00081910.

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