Subcellular Transcriptomics—Dissection of the mRNA Composition in the Axonal Compartment of Sensory Neurons

Adi Minis,[1] Dvir Dahary,[2] Ohad Manor,[3] Dena Leshkowitz,[4] Yitzhak Pilpel,[2] Avraham Yaron[1]

[1] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, 76100, Israel

[2] Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, 76100, Israel

[3] Department of Computer Science and Applied Mathematics, Weizmann Institute of Science, Rehovot, 76100, Israel

[4] Biological Services Department, Bioinformatics Unit, Weizmann Institute of Science, Rehovot, 76100, Israel

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ABSTRACT: **RNA localization is a regulatory mechanism that is conserved from bacteria to mammals. Yet, little is known about the mechanism and the logic that govern the distribution of RNA transcripts within the cell. Here, we present a novel organ culture system, which enables the isolation of RNA specifically from NGF dependent re-growing peripheral axons of mouse embryo, sensory neurons. In combination with massive parallel sequencing technology, we determine the subcellular localization of most transcripts in the transcriptome. We found that the axon is enriched in mRNAs that encode secreted proteins, transcription factors, and the translation machinery. In contrast, the axon was largely depleted from mRNAs encoding transmembrane proteins, a particularly interesting finding, since many of these gene products are specifically expressed in the tip of the axon at the protein level. Comparison of the mitochondrial mRNAs encoded in the nucleus with those encoded in the mitochondria, uncovered completely different localization pattern, with the latter much enriched in the axon fraction. This discovery is intriguing since the protein products encoded by the nuclear and mitochondrial genome form large co-complexes. Finally, focusing on alternative splice variants that are specific to axonal fractions, we find short sequence motifs that are enriched in the axonal transcriptome. Together our findings shed light on the extensive role of RNA localization and its characteristics.** Develop Neurobiol 74: 365–381, 2014

Keywords: **transcriptome; axon; RNA; high throughput sequencing; DRG**

INTRODUCTION

mRNA localization and local translation are important mechanisms by which cells spatially control their proteome. There are several advantages to this type of protein expression regulation. First, RNA localization might be energetically favorable, as small amount of mRNA molecules can be translated into

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Correspondence to: A. Yaron (Avraham.Yaron@weizmann.ac.il).

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many protein copies. Second, local translation can ensure rapid localized response to environmental cues. Third, local translation enables the cell to overcome the problem of mobilizing proteins that might be toxic during the transport (Du et al., 2007; Lécuyer et al., 2007; Besse and Ephrussi, 2008). Neurons, with their elaborated dendritic trees and long axons provide an excellent model system to study mRNA localization (Deglincerti and Jaffrey, 2012; Jung et al., 2012; Crispino et al., 2014). Multiple studies have demonstrated the importance of mRNA localization for proper dendritic function and development (Martin, 2004; Schuman et al., 2006; Bramham and Wells, 2007; Doyle and Kiebler, 2011). Yet, the appreciation of the importance of axonal mRNA localization, especially during development, has been lagging behind. Sensory neurons of the peripheral nervous system (PNS) constitute an ideal system to study mechanisms that govern axonal mRNA localization and local translation. First, these neurons extend axons to long distances from the soma. Second, unlike neurons of the central nervous system (CNS) these neurons do not have dendrites, making it a simpler model system to study. Previous work revealed that axons contain a vast repertoire of localized mRNA transcripts, some of which were demonstrated to be locally translated in response to environmental cues such as growth factors, guidance molecules, and injury (Eng et al., 1999; Spencer et al., 2000; Hanz et al., 2003; Wu et al., 2005; Leung et al., 2006; Piper et al., 2006; Jiménez-Díaz et al., 2008; Sotelo-Silveira et al., 2008; Vogelaar et al., 2009; Welshhans and Bassell, 2011; Perry et al., 2012; Cosker et al., 2013; Donnelly et al., 2013).

However, attempts to systematically study the whole transcriptome of the axon were limited to the resolution of microarrays and were thus subjected to their technical weaknesses. Importantly, it remained unclear whether the axonal transcriptome has overall unique features in terms of the mRNAs sequences and the protein functionalities encoded by these mRNAs (Willis et al., 2007; Taylor et al., 2009; Zivraj et al., 2010; Gumy et al., 2011; Deglincerti and Jaffrey, 2012).

Here we combined a novel organ culture system, which enables the isolation of total RNA specifically from the peripheral axons of mouse embryo sensory neurons, with massive parallel sequencing technology. Through this approach we determined the axonal transcriptome. High throughput sequencing provides us with a nucleotide level resolution of the transcriptome and with detailed quantification over a broad dynamic range (Wang et al., 2009; Ozsolak and Milos, 2011). Computational analysis of this transcriptome revealed various functional qualities of the axonal mRNAs, with specific protein functional classes over- and under-represented in the axon. We show differences between RNA levels and localization pattern of mitochondrial genes encoded in the nuclear *versus* mitochondrial DNA. Finally, we used a new computational approach to discover short sequence motifs that are enriched in mRNA splice variants specific to the axons, suggesting that axonal mRNAs may have common regulatory motifs. Overall, our work provides a comprehensive characterization of the axonal transcriptome.

MATERIALS AND METHODS

Antibodies

Antibodies and dilutions used for immunofluorescent staining were as follows: Neuronal class III β-tubulin (Tuj1; Covance; MRB-435P; 1:500–1:2000), SynCAM4 (Necl4; Davis/NIH NeuroMab clone N244/5; 1:400). FITC or Rhodamine Red X-conjugated secondary antibodies were used at 1:400 (Jackson ImmunoResearch Laboratories).

Mouse Strains

The Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science approved all experiments done with animals (IACUC# 02110511-1). Experiments were done with E13.5 embryos. ICR timed pregnancies were ordered from Harlan laboratories.

Medium

Neurobasal medium supplemented with 2% B-27, 1% glutamine, 1% penicillin–streptomycin, and 50 ng/mL mNGF 2.5S (Alomone Labs; N-100).

Explant Cultures

Cell culture inserts adequate to a six-well plate with 1 μm pore size were coated on both sides with Fibronectin, diluted in F12 medium to a concentration of 40 microgram/mililiter. Coating was done over night at 4°C. After rinsing the membrane, the inserts were placed upright in a six-well plate, each well containing 2 mL of medium with 50 ng/mL NGF. Spinal cords with DRGs attached or separated DRG explants were placed within the inserts and grown for 48 h, allowing the axons, but not cell bodies, to pass through the pores and grow on the bottom surface of the insert. Culture was done under normal conditions 37°C/5% CO_2.

For whole explant immune-staining, explants were cultured in three dimensional collagen matrices for 48h in the same conditions as mentioned above. Cultures were then

fixed in 4% formaldehyde and stained with anti-β-tubulin antibody, SynCAM4, and DAPI.

Quantification of Cell Nuclei

Images of DAPI stained (20× magnification), scraped and nonscraped, insert membranes were analyzed using Image J. Nuclei were detected and counted automatically using the "analyze particles" function with same parameters for all images.

RNA Extraction and Sample Preparation

For each RNA sample, SCs\DRGs were dissected from 40 E13.5 embryos and cultured as described above. Axonal and DRG explant material was collected as described by Zheng et al. (2001). Briefly, for the isolation of axonal material, the top membrane surface was scraped with a cotton-tipped applicator. Scraping was repeated three times with a fresh applicator altering the direction of scraping 90° each time. Membranes were then cut out and immersed in tri-reagent. For the DRG samples, DRGs were scraped off the top membrane surface using a cell scrapper and collected into tri-reagent. DRGs were homogenized using a 20 G syringe. This was followed by a standard tri-reagent RNA extraction protocol. All work was done in a 4°C room. In total four samples were generated—2× Peripheral axons RNA samples and 2× DRG explant RNA samples. Five micrograms of total RNA from each sample were prepared for deep sequencing following Illumina's mRNA preparation protocols. Samples were sequenced on an Illimina Genome Analyzer II instrument.

Alignment and Assembly

Each of the four samples of RNA-Seq single end 80 bases were mapped with tophat (Trapnell et al., 2012) against mm9 genome (default parameters). Transcripts assembly and quantifications were done using Cufflinks (Trapnell et al., 2012) or HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html)(htseq-count -t exon -m intersection-strict). HTSeq was done using the UCSC gene annotations. Counts from HTSeq were normalized using TMM (from edgeR package). Transcript assembly with cufflinks was done using either RefSeq (Pruitt et al., 2012) or UCSC known gene annotations (Hsu et al., 2006). Sequencing data can be found at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51572.

Functional Enrichment

Gene ontologies (GO) for mouse RefSeq genes were downloaded from Mouse Genome Informatics (MGI; www.informatics.jax.org). GO enrichments were calculated using a set of PERL and Matlab scripts (Supporting Information Files 1 and 2).

For the comparison between rat (Gumy et al., 2011) and mouse (fpkm > 10) axon localized genes we used "Homologene" database (http://www.ncbi.nlm.nih.gov/homologene) to assign orthologous genes.

Mouse orthologous for 1612 rat genes were identified and compared.

Identification of Axonal Enriched Sequence Motifs

Filtering Positive and Negative Variants. All UCSC variants were filtered according to their fpkm (fragments per kbp per million reads mapped) in the axon and DRG, where axonal variants were defined as variants with axonal fpkm >10, and DRG variants were defined as variants with DRG fpkm >1 and axonal fpkm >10. Next, genes with bilocalized variants (i.e., genes with at least one axonal variant and one DRG variant) were selected. Axonal and DRG variants of bilocalized genes were defined as positive and negative variants, respectively.

Extracting Unique Regions of Positive and Negative Variants. Using UCSC annotations, for each gene the locations of exons of its positive and negative variants were intersected, and all regions that intersect between a positive and a negative variant were removed, leaving only regions that are unique to positive or negative variants. Sequences of unique regions of both positive and negative variants were extracted from UCSC (mm9). Sequences shorter than 20 bp or longer than 500 bp were removed to eliminate biases in the motif search related to very short or long sequences, and to facilitate the computational motif search.

Identification of Sequence Motifs in the Unique Regions. The FMM tool (Sharon et al., 2008) (default parameters, top five motifs reported) was used to search for short sequences that are enriched in the unique positive regions compared to the unique negative regions. Data were divided in a fivefold cross-validation (CV) scheme, and for each CV fold, motif search was performed only on the training set. The FMM tool was also used in fivefold CV on a permuted positive set (with the original negative set), where every positive sequence was permuted while maintaining nucleotide composition. Motif match scores on the held-out test set were computed using the PSSMs output of the FMM tool, with a background model of single nucleotide frequencies in the data (of both positive and negative variants).

RESULTS

Isolation of Peripheral Axons from Dorsal Root Ganglia Sensory neurons

Previous studies have used dissociated cell cultures in order to isolate axonal RNAs (Zheng et al., 2001). This type of culture has two disadvantages. First, the architecture of the neuronal ganglia and its

connections are destroyed. Second, the identity of the axons from which the RNA is isolated is not clear. In order to overcome these drawbacks we established a novel culture system for embryonic dorsal root ganglia (DRG) sensory neurons. DRG neurons have a pseudo unipolar morphology, with two processes, both of which express axonal markers. Nevertheless, these two axons are functionally distinct. One axon extends to the periphery, where it ends in a sensing organ, while the other goes into the spinal cord and synapses on neurons in the central nervous system [Fig. 1(A)]. We dissected E13.5 mouse embryos DRGs while they are still attached to the spinal cord. Cultures of these organs in the presence of nerve growth factor (NGF) elicit strong axonal growth of the peripheral axons out of the attached DRGs, while the central axons remain attached to the spinal cord [Fig. 1(B,C)]. Importantly, we did not observe any axonal growth in cultures of the spinal cord alone [Fig. 1(D)], demonstrating that in our system all the axons that grow out are DRG peripheral axons. Moreover, since we supplement the medium only with NGF and not with other neurotrophic factors, we select for axonal growth from a particular subset of DRG sensory neurons (Lallemend and Ernfors, 2012). Next we established this novel organ culture on insert membrane, which was previously used to isolate axonal extracts (Zheng et al., 2001; Schoenmann et al., 2010) [Fig. 1(E)]. We then performed a cellular analysis of the membrane to validate that only axons manage to cross the membrane to the bottom part and that there is no contamination by cell bodies or cell processes, especially glial cells. For this we have stained the membrane before and after clearing of the upper side, where the spinal cord explant is placed, for axons (by anti-Tuj-1), Glia (by anti-Necl4 (Spiegel et al., 2007)), and nuclei (by DAPI). While before scrapping we clearly detected axons, glia cells and cells nuclei (313 $\pm$ 95 nuclei per image, $n = 4$), after clearing of the upper side only axons could be detected (0 $\pm$ 0 nuclei per image, $n = 6$) [Fig. 1(F)]. Therefore, using this culture system we can obtain highly enriched axonal material. This combination enabled, for the first time, specific isolation of peripheral axons of embryonic DRG neurons. In addition, we extracted RNA from the cellular part of DRG cultures in the upper part of the insert which we referred to as DRG. Poly-adenylated RNAs of each part were isolated and sequenced using the Solexa platform. Each RNA sample was generated from multiple cultures and therefore represents a RNA pull of many embryos. Our analysis is based on data collected from two independent replicates of RNA purification and sequencing.

Unique Functional Composition of the Axonal Transcriptome

In order to characterize the differential localization of mRNAs between the axon enriched RNA and the DRG, we derived the mRNA abundance of each gene in each fraction applying the Tophat-Cufflinks and Tophat and HTSEQ (Trapnell et al., 2012, http://www-huber.embl.de/users/anders/HTSeq/doc/count.html).

This resulted in two fpkm (fragments per kbp per million reads mapped) values per transcript, representing its relative mRNA levels in the axon and DRG, respectively.

Importantly, correlations of expression values between biological duplicates were very high ($R^2_{DRG} = 0.98$ and $R^2_{Axons} = 0.99$) demonstrating the strong reproducibility of our procedures (Supporting Information Fig. S1A).

To assign each gene with a value depicting its enrichment or depletion in the axonal fraction, we divided its axonal fpkm value by its total fpkm (axon + DRG) and defined it as the *diff* value of the gene. The *diff* values can span the range from 0 (i.e. depleted from axons) to 1 (highly enriched in axons). Only genes with fpkm >1, in at least one of the datasets, were included in subsequent analyses (this threshold was picked after examining the mRNA expression levels of genes that are specific to sperm cells which are not supposed to be expressed in neurons (Yamashita et al., 2008; Liu et al., 2011). This threshold resulted in a total of 12,605 genes for which we could compute a *diff* value. We used this low threshold of fpkm >1 to uncover significant, enriched or depleted, protein functional categories in the axonal transcriptome. In addition, we used a more stringent criterion of fpkm >10 when we examined specific genes. This latter dataset is comprised of 7387 genes that are at least moderately found in either the axons or the DRG, of which 6118 genes are localized to the axons with mRNA levels of fpkm >10.

Figure 2(A) shows the distribution of *diff* values over the 12,605 analyzed genes. Interestingly, while most of the analyzed mRNAs were represented in the axon and the DRG fractions with similar amounts, depicted by *diff* values around 0.5, many genes show either strong enrichment or depletion from the axonal mRNA population (high and low *diff* values, respectively). Reassuringly, more than 80% of the genes whose mRNAs were found in rat embryonic axons by Gumy et al. (2011) also passed our stringent criterion for axon localization with fpkm >10 [Fig. 2(B)]. As expected from the high sensitivity of deep sequencing compared to microarray, we detected mRNAs of

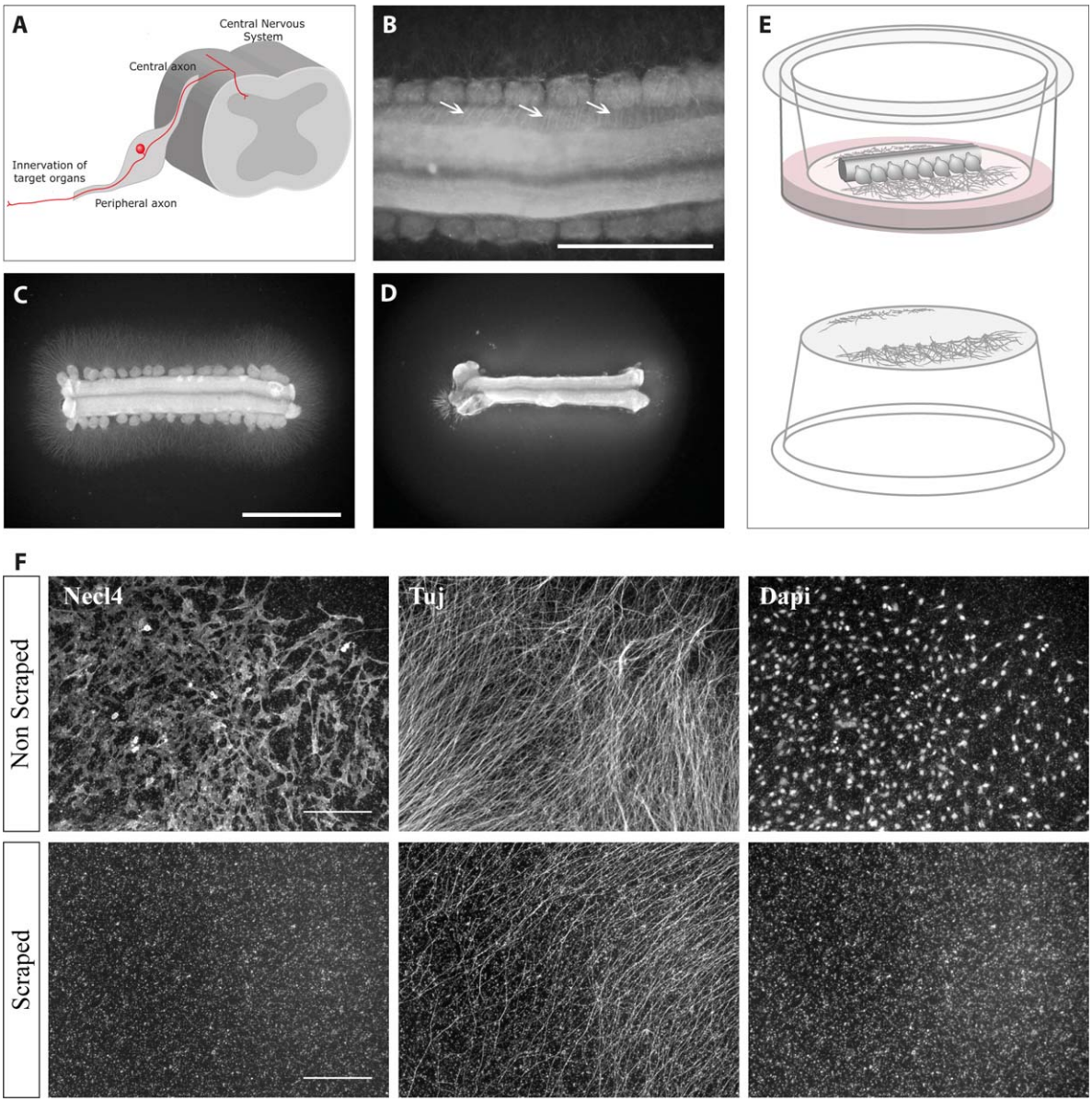


Figure 1 Explant culture and peripheral axons isolation. (A) DRG sensory neurons are pseudounipolar cells that grow a long axon that bifurcates into two main processes. One process elongates toward the peripheral target organ while the other one elongates toward the central nervous system. (B) CNS oriented axons of DRG neurons stay connected to the spinal cord in culture. Arrows point to central axon bundles. (C, D) Spinal cord explants with (C) or without (D) attached DRGs, cultured for 48 h in the presence of NGF. Axons grow out of the DRG neurons but not out of the naked spinal cord. (E) Insert-membrane system. Spinal cords with DRGs attached to them were cultured on 1 μm pore membranes in the presence of NGF for 48 h, allowing peripheral axons to grow through the pores, while keeping the cell bodies above. Tissue was then removed from the upper part of the membrane, and axons remaining on the lower surface were harvested for RNA extraction. Scale bars, 200 μm. (F) Immunostaining of insert membrane before and after scrapping the spinal cord explant culture from the upper side of the membrane. Staining for Necl4 (glia), Tuj1 (neurons), and DAPI (nuclei) is prominent before scrapping the membrane, while only Tuj1 staining is still visible after scrapping clean the upper side of the membrane. Membrane pores are non-specifically stained in all of the pictures—notice the size difference between pores and nuclei. Scale bars, 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

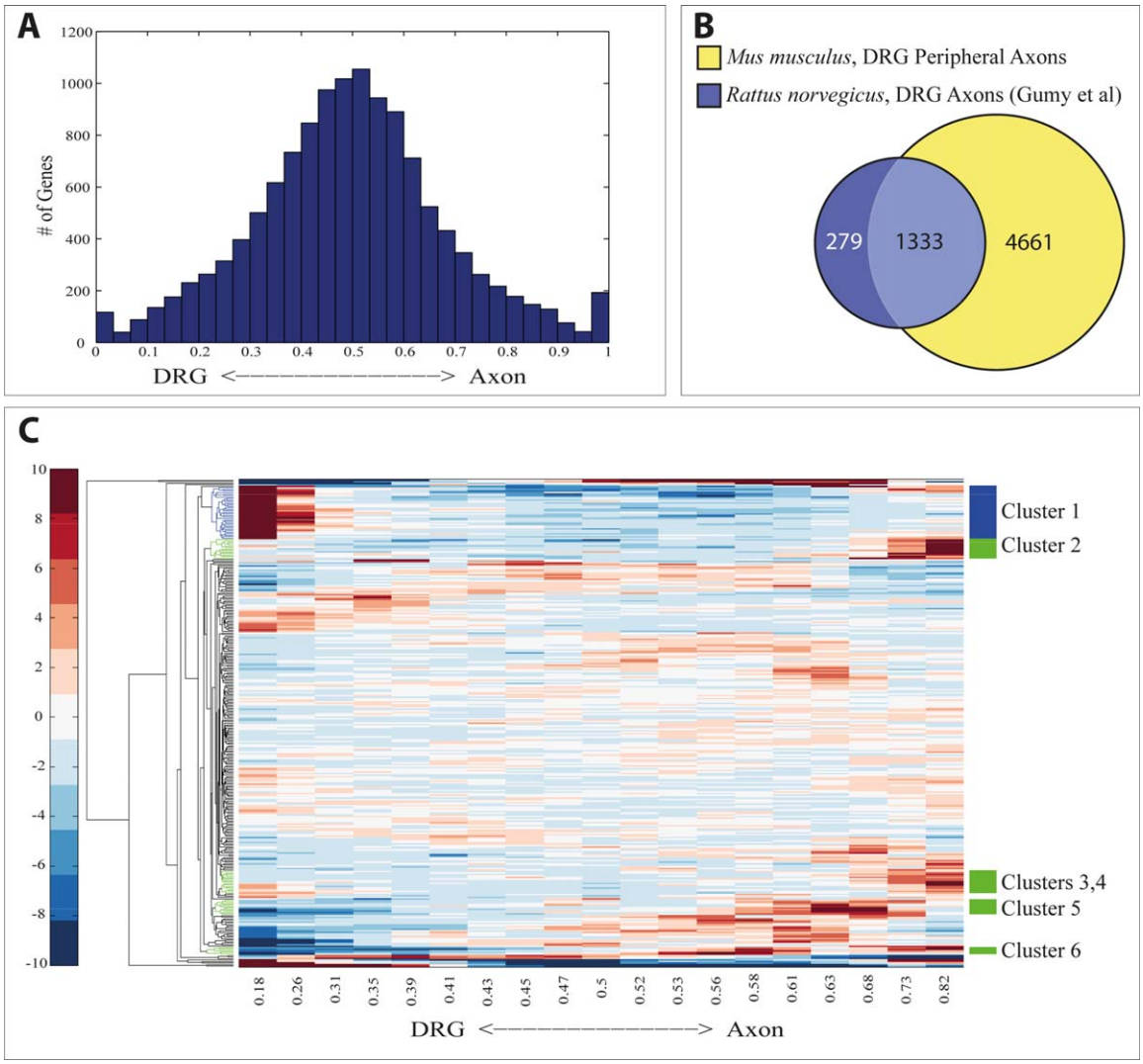


Figure 2 Differential mRNA levels in dorsal root ganglia and peripheral axons. (A) A histogram describing the distribution of *diff* values over 12,605 genes mRNA level in either DRG or peripheral axons. *diff* is defined as the gene axonal fpkm value divided by its total fpkm (axon + DRG). Genes with *diff* >0.5 are enriched in the axons, while those with *diff* <0.5 are enriched in the DRG. (B) Venn diagram comparing genes localized to peripheral axons (with stringent threshold of fpkm >10) and genes that are localized to rat embryonic DRG axons (Gumy et al., 2011). (C) Enrichment in Gene Ontology categories by differentially localized genes. The mRNAs expression dataset (fpkm >1) was divided by *diff* values into 19 overlapping bins; each contains 10% of the data (*x*-axis). Each bin was then tested for the enrichment or depletion of any GO category (*y*-axis). The GO categories are color-coded by the significance (−log (*p*-value)) of their enrichment (red and blue depict enrichment and depletion, respectively), clustered and plotted over the bins. Green and blue bars mark GO clusters that are enriched or depleted from the axons respectively. Detailed diagrams corresponding to the annotated clusters 1–6 are included as Supporting Information Figures S2 to S7. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

almost two times more genes in the axons than Gumy et al. (2011). To examine the data in terms of functional categories of the encoded proteins, their biochemical features and their cellular localization, we used the Gene Ontology (GO) annotations available for the genes in our dataset. To this aim we first divided the dataset, sorted by *diff* values, into 19 overlapping bins, each comprising 10% of the data. We then looked for enrichment or depletion of different GO categories in each of these bins (for this analysis we used GO categories that were associated with at least 50 genes that are expressed in our dataset).

Figure 2(C) illustrates the enrichment or depletion of each such GO category in each of the 19 overlapping bins of the data. As expected from the distribution of the *diff* values, many GO categories do not show any statistically significant enrichment or depletion in any of the bins. Still, for several clusters of functional and biochemical categories, we found highly significant differential localization of mRNAs between the axon and the DRG fractions. GO categories corresponding to "translation," "sequence specific DNA binding", "extra cellular matrix," and "immune response" are found enriched in the bins representing axon-enriched genes [Clusters 2–6 in Fig. 2(C), Supporting Information Fig. S3–S7]. In addition, this comparison also highlights the GO categories of mRNAs that are depleted from the axons [Cluster 1 in Fig. 2(C), Supporting Information Fig. S2], which the most striking one is of membrane proteins. It should be noted that the "DRG" sample includes cells that are non-neuronal, such as glia and fibroblasts. Thus, in order to minimize our error, we focused our analysis on categories that are in the two extremes of the *diff* values—either enriched or depleted (corrected p values $<$1e-05) from the axonal mRNA population.

Membrane-Associated Proteins

One of the most intriguing observations is the axonal depletion of genes under the GO category "membrane fraction" ((GO:0005624), p value = 1.3e-09; 2.2e-05, Bonferroni corrected) (Supporting Information Fig. S2). Remarkably, although many of these proteins are mostly found in axons and specifically in synapses (GO:0030424 and GO:0045202, respectively, Supporting Information Fig. S2), their mRNAs are strongly depleted from the axons. To further examine this observation we specifically looked into the localization of mRNAs for neurotropic factors and axon guidance receptors. These two groups of receptors are known to function at the tip of the axon as it navigates towards its final destination during development (Huber et al., 2003; Kolodkin and Tessier-Lavigne, 2011). In accordance with the overall behavior of the membrane associated proteins mRNAs, we detected a strong depletion of most of these receptors-encoding mRNAs from the axons [Fig. 3(A,B)].

Therefore, although many membrane proteins function in the axon, the cell mainly utilizes other means such as protein transport, membrane addition or vesicles transport to bring them to their destination.

In contrast to the results we obtained with mRNA for trans-membrane (TM) proteins, we have found a strong axonal enrichment of multiple mRNAs for secreted proteins (Supporting Information Fig. S3). Both, transmembrane and secreted proteins are targeted to the Endoplasmic Reticulum (ER) on their way to the plasma membrane where they will reside or be secreted from the cell, respectively. Therefore we carefully tested if the mRNAs for the different types of trans-membrane proteins are equally depleted from the axons.

Interestingly, we found that while mRNAs that encode multi-TM domains that also contain SP motif proteins were strongly depleted from the axonal fraction, the extent of this depletion is milder for mRNA of type I TM proteins (with TM and SP) and even weaker for type II TM proteins that do not contain the SP motif [Fig. 3(C)]. As noted above, we have detected many axonal enriched mRNAs for secreted proteins that contain only the SP motif.

One interesting group of secreted proteins, whose mRNAs are highly enriched in the axons, are the extracellular matrix proteins of the collagen family. The extra cellular matrix (ECM) plays an important role in the development of the peripheral nervous system and was shown to play a crucial role in the myelination of axons in the peripheral nervous system (Podratz et al., 2001). Proteins from the collagen family are essential members of the ECM. Since in their mature form they are nonsoluble proteins, collagens are transported as pre-proteins and polymerized locally outside of the cell. Moreover, due to their large size, they are mobilized trough a complex transport system in vesicles that are larger then typical (Prockop and Kivirikko, 1995; Venditti et al., 2012). One way to minimize the distance of this complex transport of pre-collagen molecules to the ECM is to locally translate them at the point of secretion. In accord with this notion, we detected axonal enrichment for multiple mRNAs for members of the collagen family [Fig. 3(D)]. Interestingly, the modifying enzymes of the collagens show a similar behavior [Fig. 3(E)]. This may suggest that axons are locally translating and secreting these proteins to modify the ECM as they grow in it. mRNAs for other members of the collagen family were depleted from the axon albeit strongly detected in the DRG. This might reflect the usage of different ECM compositions in distinct anatomical regions.

mRNAs of Transcription Factors—Axon-Nuclear Communication

Additional GO categories that were enriched in axons are nuclear proteins and DNA binding proteins

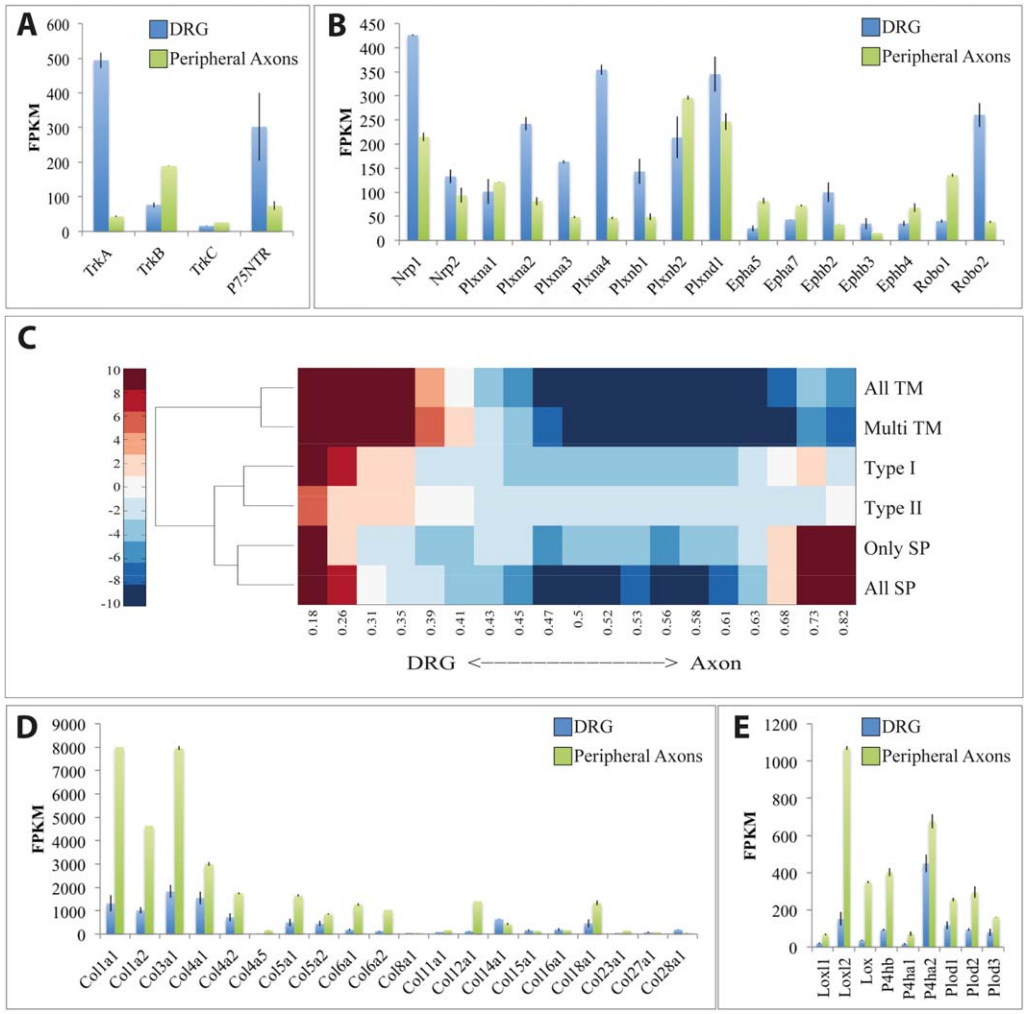


Figure 3 Distribution of mRNAs for membrane associated and secreted Proteins. (A, B) mRNA levels by fpkm of membrane receptors that have a role in (A) neuronal survival and (B) axon guidance. (C) Enrichment map of GO categories for membrane associated proteins. The mRNAs expression dataset (fpkm >1) for membrane and secreted proteins was divided by *diff* values into 19 overlapping groups, each contain 10% of the data (*x*-axis). Each group was then tested for the enrichment or depletion of specific GO categories for membrane associated proteins (*y*-axis). The GO categories were then color-coded by the significance (P) of their enrichment (red strong enrichment to blue strong depletion), clustered and plotted over the *diff* groups. While the GO categories for transmembrane proteins are strongly depleted from the axons and enriched in the ganglia, the GO categories for proteins with signal peptide (SP) are axonally enriched. (D, E) mRNA levels by fpkm of members of the collagen family (D) and the collagen biosynthesis pathway (E). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Supporting Information Fig. S3). Although surprising, this observation is in agreement with recent reports that found mRNAs for transcription factors (TFs), the nuclear transport machinery and the nuclear envelope in axons (Hanz et al., 2003; Cox et al., 2008; Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012; Yoon et al., 2012). To learn more about this growing group of potentially locally translated TFs we surveyed the mRNA expression of several TF families, some of which were reported to localize in axons, and to convey axon-nuclear communication (Fig. 4). In line with previous reports (Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012), we have detected the axonal localization of *Stat3* but also of *Stat1* and mRNAs for two members of the Smad family (*Smad4* and *Smad5*) [Fig. 4(A)]. Next we examined the mRNA localization of several TFs that play a key role in sensory neurons fate decisions.

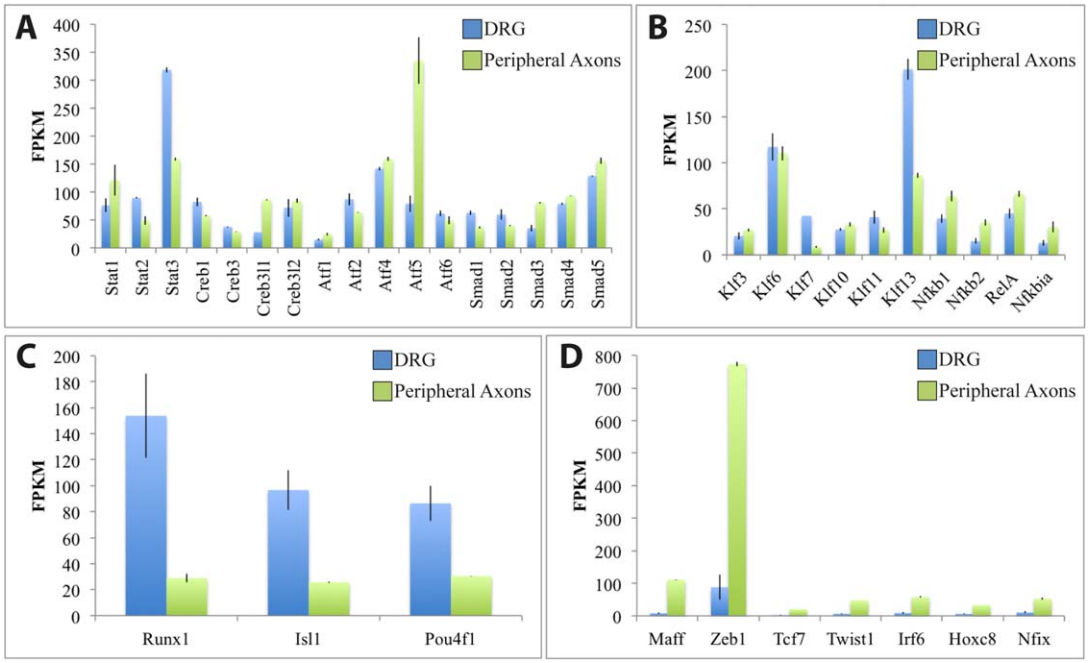


Figure 4 Axonal localization of mRNAs for transcription factors. (A, B) mRNA expression levels by fpkm of TFs which were previously reported to reside in the axon (A) or to have an affect on axon growth and maintenance (B). (C) mRNA expression levels by fpkm for TFs involved in sensory neurons fate determination. (D) mRNA expression levels by fpkm of TFs that are strongly enriched in axons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In agreement with the fact that we selected for nociceptive neurons in our culture system as we supplied it only with NGF, we have detected high mRNA levels of the TFs *Islet1, Brn3a*, and *Runx1* (Lallemend and Ernfors, 2012). Importantly, the mRNA levels were very high in the ganglion but well below our threshold for axonal localization [Fig. 4(C)]. This is in line with the high mRNA expression of TrkA—NGF receptor, which its mRNA like the mRNAs for other transmembrane proteins, is also depleted from the axons [Fig. 3(A)].

We then turned to examine the mRNA levels and localization of TFs that are known to regulate axonal growth—the Klfs and members of the Nf-kB family (Meffert et al., 2003; Gavaldà et al., 2009; Moore et al., 2009). Members of the Klf family have been shown to promote (*Klf6* and *Klf7*) and suppress (*Klf4* and *Klf9*) axonal growth of retinal ganglion cells. In agreement with these roles, we have detected very low mRNA levels of *Klf4* and *Klf9* in embryonic DRG and high levels of *Klf6* and *Klf7* [Fig. 4(B)]. Interestingly, only *Klf6* demonstrated a strong axonal localization, suggesting it may activate a growth-promoting program in response to environmental cues through axonal local translation and retrograde transport. The Nf-kB family of TFs has also been shown to regulate axonal growth and synaptic signaling. The localization of mRNAs for these TFs in axons has been reported. We detected mRNAs for several members of this family including high levels of *RelA* (*p65*), *Nfkb1* (*p105*) and *Nfkb2* (*p100*) [Fig. 4(B)]. Interestingly, the most abundant mRNA is of *IkB*, the inhibitor of *Nf-kB* that prevents its nuclear translocation. Although this seems surprising, one should keep in mind that *IkB* is degraded upon *Nf-kB* activation. Therefore, its local replenishment may be achieved by local translation, and not transcription as happens in the cell body. Our analysis also discovered several TFs whose mRNAs were strongly enriched in the axons, most prominently *Maff* and *Zeb1* [Fig. 4(D)]. Interestingly, *Maff* is an early NGF responsive gene in PC12 cells and *Mafk*, a close homolog of *Maff*, has been implicated in NGF induced axonal growth in these cells (Töröcsik et al., 2002). The role of *Zeb1* in the development of the nervous system is not known. However, its *Caenorhabditis elegans* homolog, Zag-1, controls multiple aspects of axonal behavior including guidance, branching and fasciculation (Clark and Chiu, 2003; Wacker et al., 2003). Overall, our survey shows the existence

of mRNAs for multiple TFs in axons, and suggests that TFs that were previously shown to regulate axon behavior may be controlled by axonal local translation.

Mitochondrial Transcriptome

We next turned to examine mRNAs encoding for mitochondrial proteins. Most of the mitochondrial proteins are encoded in the nuclear DNA, yet, some key proteins are encoded in the small mitochondrial genome. The great physical distance between nuclear DNA and the mitochondrial DNA in axons prompted us to examine how the two mRNA pools behave in relation to each other. This question is more intriguing when taking into account that many of the mitochondrial proteins should form large protein and ribo-protein complexes such as the electron transport complexes and the mitochondrial ribosome.

First, we assessed the mitochondrial DNA (mt-DNA) encoded RNA. More than 20% of the total quantity of mRNA transcripts in the axon aligns to RNA from the mitochondrial chromosome, while in the DRG they comprise only 2%. Most mRNAs transcribed from the mt-DNA are enriched 3- to 10-fold in the axons. Furthermore, mitochondrial ribosomal RNA is extremely enriched in the axon with fpkm levels over 50-fold higher in the axons than in the DRG. The differences in RNA levels are clearly demonstrated by deep sequencing read coverage of the mt-DNA encoded genes [Fig. 5(A)]. We then examined mRNAs for the nuclear encoded mitochondrial proteins. Surprisingly, most of these mRNAs were represented in similar levels in the two cellular compartments [Fig. 5(B)]. Moreover, the RNA levels of the nuclear encoded mitochondrial genes were 1 to 2 orders of magnitude lower than those encoded in the mitochondrial chromosome.

Overall, our analysis uncovers surprising relationship between the two transcriptomes that encode the mitochondrial proteome.

Identification of Axonal Enriched Sequence Motifs

Previous studies too have reported the existence of multiple axonal and dendritic mRNAs (Poon et al., 2006; Willis et al., 2007; Taylor et al., 2009; Zivraj et al., 2010; Gumy et al., 2011; Cajigas et al., 2012). However, whether these mRNAs share common motifs remained an open question. We first approached this problem using the motif finding algorithm "Feature Motif Model" FMM (Sharon et al., 2008) to search for sequence motifs that are enriched in axon-localized mRNAs. For this analysis we first used the RefSeq gene annotations based transcript assembly. However, we were not able to uncover any common motif that is statistically more significant than motifs found in a permuted data set that contains the same nucleotide composition (data not shown). In order to improve the signal-to-noise ratio we tried a different computational approach, taking advantage of the fact that using high throughput sequencing we could quantify different splice variants of each gene (termed here *variants*). In addition, we used UCSC gene annotations based transcript assembly since UCSC annotations are more comprehensive than RefSeq and include more variants for differentially spliced genes (see materials and methods for details). Specifically we assembled a set of genes for which at least one splice variant is preferentially axonal mRNA, and at least one variant that is absent from the axons but present in the DRG (threshold for mRNA presence was set to 10 fpkm in axons and 1 fpkm in DRG). This resulted in a set of 1556 genes with 4169 transcripts. In order to search for enriched sequence motifs in axonal mRNAs we have divided all transcripts in the set into two groups, wherein variants that are localized to the axons are termed "positive variants" and the ones that are not localized to the axons are termed "negative variants." This resulted in 1888 and 2281 positive and negative variants, respectively. Since for any given mRNA, most of its sequence is shared across different variants, we hypothesized that a region shared by both positive and negative variants is not likely to harbor an axonal motif. Therefore, we removed all overlapping sequences between the positive and negative variants of each gene. In addition, we filtered these unique regions to a minimum/maximum length of 20/500 bp to facilitate the motif search. This resulted in 7743 and 6202 unique sequences for positive and negative variants, respectively. We divide the positive and negative sequences into fivefold cross-validation (CV) disjoint partitions for further analysis. Next, we used FMM (Sharon et al., 2008), a discriminative sequence motif search tool to find short sequence motifs that are enriched in the positive sequence set *versus* the negative sequence set. As a control, we permuted the positive sequences (i.e. maintaining the same nucleotide composition for each variant), and re-ran the FMM on the permuted positive sequences *versus* the original negative sequences. We found that when FMM ran on the original data, the same short sequence motifs were robustly enriched for different CV partitions [Fig. 6(B), Supporting Information Fig. S8A], in contrast to the permuted data, where for each CV partitions different motifs were

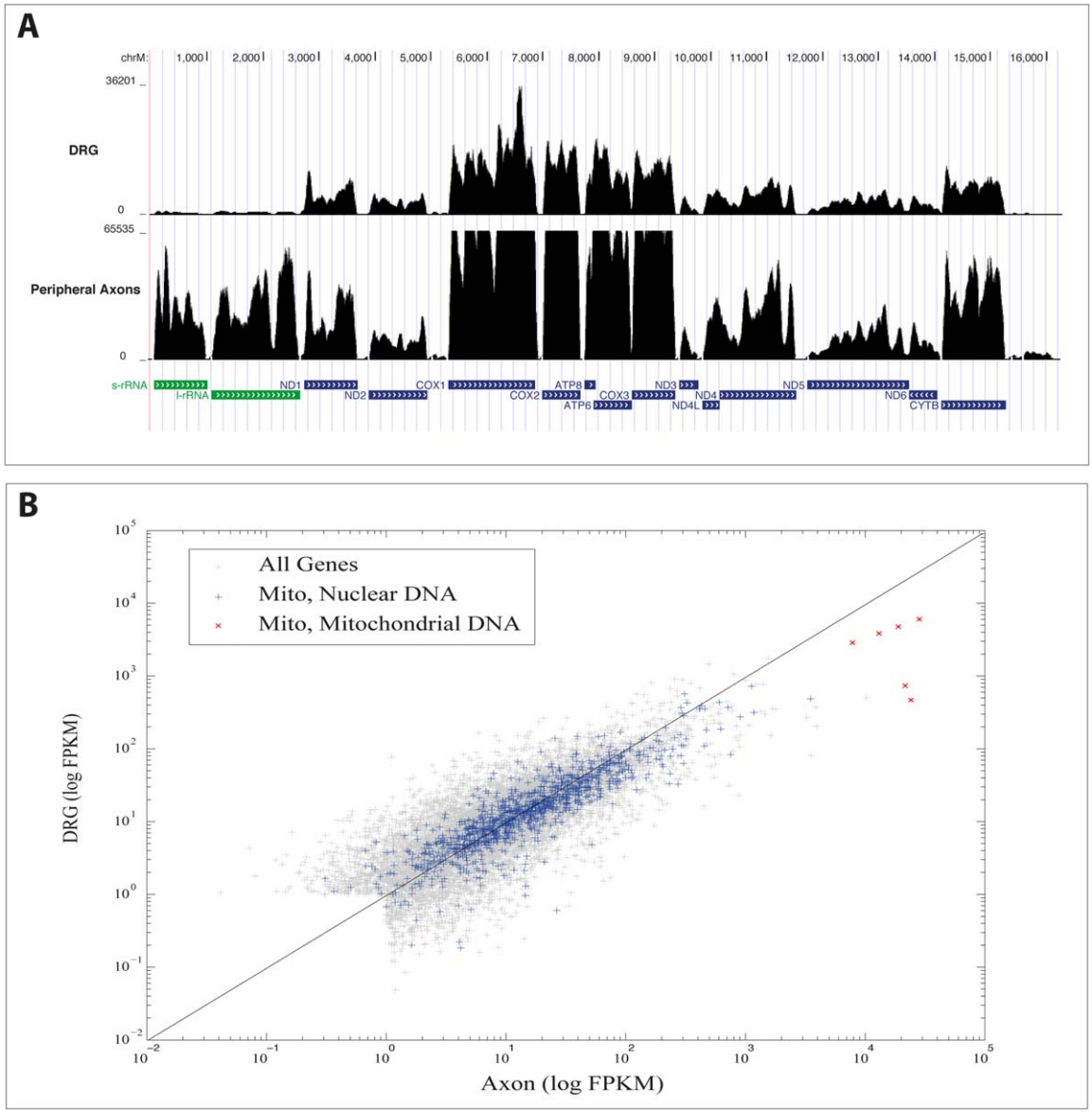
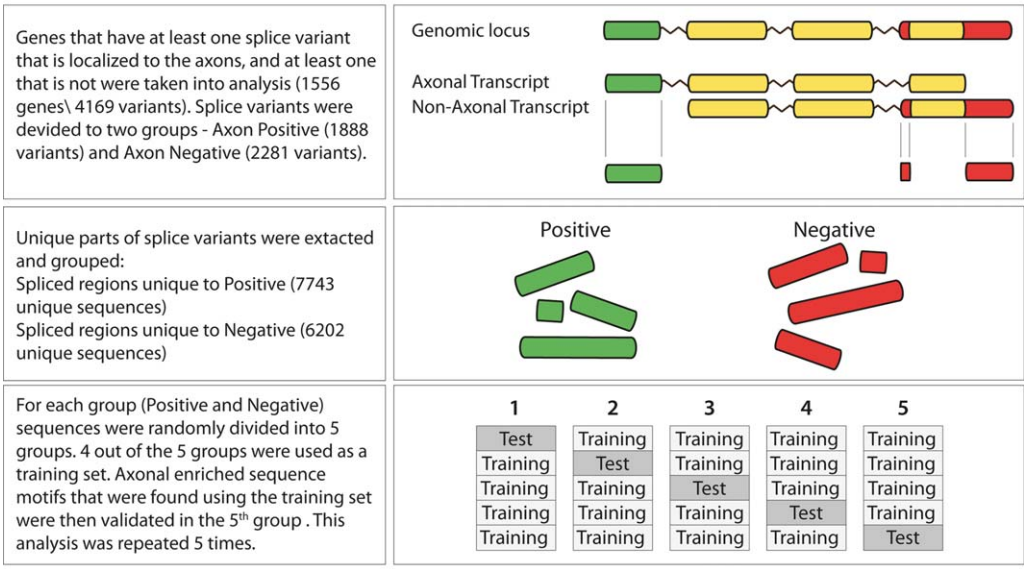


Figure 5 Distribution of the Mitochondrial mRNAs (A) UCSC genome browser diagram depicting deep sequencing reads aligned to the mitochondrial chromosome. The maximum number of reads mapped to the locus is shown in the *y*-axis. Much higher RNA levels of all mitochondrial genes are detected in the axons with the most dramatic difference seen in the mitochondrial rRNAs (s-rRNA and l-rRNA) marked in green. (B) Distribution of the mitochondrial mRNAs in comparison to the entire transcriptome. Axon (*x*-axis) *versus* DRG (*y*-axis) mRNA expression levels of the entire transcriptome (gray), nuclear encoded mitochondrial genes (blue) and mitochondrial encoded mitochondrial—mt-DNA (red). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

found (i.e., they are not robust, Supporting Information Fig. S8B). In addition, although there is a small GC% bias toward the positive sequences (GC content is higher in positive sequences), the motifs found in the real data were not GC rich [Fig. 6(B), Supporting Information Fig. S8A], compared to the motifs found on the permuted data that were all GC rich (Supporting Information Fig. S8B). As we used cross-validation, we found for each held-out test case its best hit for the motifs found in the training set. We find that the motifs found in the real data have a significantly higher score on real test data than the motifs found in permuted data on the permuted test data (median p value $<10^{-44}$ using t-test to compare

A



B

Motif	Consensus	# of Instances across CV groups
1	CTG(G/A)ANNNNCTG(G/A)A	3/5
2	ACCTGNNNNACCTG	3/5
3	AAGAAG	5/5
4	GCTGCTG	3/5
5	ATGACAA	3/5

C

Motif	TM Score (AU)	SP Score (AU)	P-Value	SP/TM Score
1	0.00350	0.00507	1.2912e-60	1.44
2	0.00337	0.00475	2.3976e-51	1.40
3	0.00339	0.00515	5.2518e-58	1.51
4	0.00286	0.00394	8.6384e-32	1.37
5	0.00334	0.00473	5.2103e-29	1.41

Figure 6 Sequence analysis of mRNA splice variants reveals short consensus sequences that are enriched in axonal transcripts. (A) Pipeline of the bioinformatics sequence analysis. (B) A table describing five short sequence motifs enriched in axonal transcripts. These motifs appeared repeatedly in different CV partiotions. (C) A table comparing best sequence match of the above-described motifs between transmembrane proteins and secreted proteins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

test score distributions across five different permutations). As a totally independent validation of the motifs, we took only the positive variants, and found for each one the best motif hit (in its spliced unique regions). Next, we checked whether the variants with motif score in the top 20%, have also significantly higher mRNA levels in the axon. We found that 7/25 motifs found for the real training data across different CVs show this significance (Wilcoxon rank-sum test p value <0.05), compared to $\sim$1/25 motifs found for permuted training data, strengthening the significance of the motifs. Overall, our analysis implies that axonal mRNAs do share relatively unique common short sequence motifs [Fig. 6(B)].

These results and our findings that mRNAs for transmemebrane proteins are strongly depleted from the axons unlike the mRNAs for secreted proteins, promoted us to test whether there is a correlation to the presence of the motifs in these two groups of mRNAs. Interestingly, we found significant

enrichment of the axonal motifs in the mRNAs for secreted proteins over those for transmemebrane proteins [Fig. 6(C)]. This demonstrates that the presence of the axonal enriched motifs can be correlated with axonal enriched functional groups.

Since the common notion today is that RNA localization signals are mainly located in the 3′UTR of an mRNA sequence (Kislauskis et al., 1993; Andreassi and Riccio, 2009; Andreassi et al., 2010; Perry et al., 2012), we have examined the motifs locations along the genes that were analyzed. To this end, we looked for the best hit of each motif and checked if it is located in the 5′UTR, CD or 3′UTR of the gene. We have found that the best motif hit is found 4% of times in the 5′UTR, 3% in the 3'UTR and 93% in the CDS. However, the overall fraction of base pairs in the different regions across all variants is 7.5%, 5.5%, and 87% for the 5'UTR, 3'UTR, and CDS, respectively. Therefore, it doesn't seem that there is a clear enrichment for one of the regions (5'UTR, CD, or 3'UTR) for the appearance of the best motif hit. Last, using the same analysis in order to find DRG enriched sequence motifs, ended with two consensus sequences (Supporting Information Fig. S8C).

DISCUSSION

The Axonal Transcriptome and Proteome

Axons are constantly modifying their protein content (proteome) as they grow to their targets, making synapses or in response to injury. Which part of this proteome is controlled by local translation and which part by active protein transport from the soma, is still under debate. Still, there is growing evidence that localized transcriptomes might be much larger than was predicated before. While previous reports have identified about 200 different mRNAs in dendrites, a recent study using high throughput sequencing suggested that there are 10 times more dendritically localized mRNAs (Poon et al., 2006; Zhong et al., 2006; Cajigas et al., 2012). Our work largely extends the list of mammalian axonal mRNAs, suggesting that axons contain a highly enriched repertoire of mRNAs than previously assumed both for mammalian cortical and somatosensory axons (∼300 and ∼3000 mRNA transcripts, respectively) (Taylor et al., 2009; Gumy et al., 2011). Due to the high sensitivity of our detection method one cannot completely rule out that some of the axonal RNAs that we uncovered are due to contamination by the DRG RNA. However, it is noteworthy, that many genes and the main gene groups that are enriched in the above-mentioned studies, i.e., genes related to translation, mitochondria, cytoskeleton and intracellular trafficking, are substantially represented in our axonal transcriptome as well.

It is conceivable to think that all the proteins that are mainly functioning within the axon will be locally translated, due to probable energetic advantages. However, our axonal transcriptome analysis clearly shows that this is not the case. Our findings clearly demonstrate that mRNAs for TM proteins, many of which function at the tip of the axons, are largely depleted from the axons. This suggests that these proteins are constantly mobilized from the soma either by vesicular transport or by lateral translocation along the plasma membrane. What is the advantage of TM protein transport over local translation? One explanation might be the complex process TM proteins undergo during their maturation, which includes folding in the ER, insertion to the membrane and many post-translation modifications. Indeed, the nature of the axonal ER is not clear and whether it contains all the elements of the somatic ER is still an open question (Brittis et al., 2002; Merianda et al., 2009). Therefore, it might well be that axons, in the embryonic growing phase, contain a simpler version of the ER which do not allow them to efficiently handle translation of complex TM proteins. It should be noted that axonal transcriptomes from adult animals did report the existence of many mRNAs for trans-membrane proteins (Zheng et al., 2001). This argues that the ER can mature with the axon, allowing the local translation of more complex proteins in later developmental stages.

The presence of TF mRNAs and their local translation was demonstrated by previous work in the field (Cox et al., 2008; Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012). We would like to add another possible aspect to the role these TFs might play in the axons, in addition to their retrograde signaling to the nucleus. Recent findings suggest that some TFs [Stats, Creb, Nf-kB, see Fig. 4(A,B)] might also work in the mitochondria by affecting mt-DNA encoded RNA transcription and even in nontranscription related ways (Cogswell et al., 2003; Lee et al., 2005; Gough et al., 2009; Szczepanek et al., 2012). Since mitochondria play an important role in both axon elongation and maintenance, local synthesis of these TFs might be important in the local regulation of mitochondrial signaling and energy homeostasis.

TFs were also shown to have a non-transcriptional role outside of the mitochondria. In a recent work by Selvaraj et al., it is shown that activated STAT3 can locally stabilize microtubules in motoneurons axons, thus promoting axon elongation and maintenance. STAT3 exerts this effect by directly interacting with Stathmin and inhibiting its microtubule-destabilizing

activity (Selvaraj et al., 2012). Thus, some TFs may be locally translated in the axons to operate in non-transcriptional mode.

Mitochondrial Genes—Local (mtDNA) and Distal (nDNA) Transcription Sites

In this study we examined, for the first time, the relationship between the mitochondrial transcriptome and nuclear encoded mitochondrial genes. We found a significant enrichment of mRNAs encoded in the mitochondrial genome in the axons. This was not the case for nuclear encoded mitochondrial genes that tend to be represented similarly in the two compartments. Moreover, the difference between the two gene groups is enhanced when looking at the mRNA levels. Since the proteins from the two transcriptomes must form joint functional complexes and the mitochondria encoded rRNA form a complex nuclear encoded proteins, these differences should be further studied. One explanation for this observation may be that the mRNA stability of the two groups is different. In this case, nuclear encoded mitochondrial genes might need less mRNA molecules and may undergo enhanced translation or that the local translation is backed-up by protein transport. Another point to consider is the morphology of the mitochondria. In the cell body, mitochondria tend to fuse and form very long, tubular structures. In contrast, in the axon, which is narrow and long, mitochondrial units are visible, but they are much shorter (Popov et al., 2005). Proteins encoded in the nucleus may be translated and exported to each of these axonal mitochondria, however, proteins that are encoded in the mitochondria, serve only the mitochondrion in which they were generated. Hence, more mitochondrial units, even though each one of them is smaller, will need more mRNA molecules than one large mitochondrion.

Further, proteins encoded in the mitochondria might have different dynamics than those encoded in the nucleus. It could be that these genes provide evolutionary advantage if maintained in the mitochondrial genome, allowing mitochondria to respond to their environment. Local translation of mitochondrial proteins has been demonstrated to affect mitochondrial membrane potential, ATP production and reactive oxygen species generation (Kaplan et al., 2009; Aschrafi et al., 2010; Natera-Naranjo et al., 2012; Rugarli and Langer, 2012).Thus, the mRNA enrichment and different composition (dramatically illustrated by the mitochondrial rRNA) between mitochondrial genes in the axon and in the cell bodies may reflect the different needs of each of these compartments.

Axonal Enriched Short Sequence Motifs

The present study identifies, for the first time, putative axonal mRNA motifs. Although previous studies have uncovered mRNA elements, especially in the 3'UTR, which are required for mRNA axonal targeting, no consensus element for multiple mRNAs has been reported. It was argued that this might be due to such elements being based on structure and not on primary sequence. Our identification of putative axonal mRNA motifs was made possible by our identification of specific splice variants in axons, which was never done before. This might suggest a connection between splicing and axonal targeting. The connection between mRNA targeting and alternative splicing was best demonstrated in *Drosophila Melanogaster*, where the mRNA for *Oskar* is selected for targeting during the splicing process (Hachet and Ephrussi, 2004; Ghosh et al., 2012). Moreover, previous studies have shown that specific mRNA splice forms are targeted to the axon (Yudin et al., 2008; Andreassi et al., 2010; Perry et al., 2012). However, this connection was never demonstrated at the level of the whole transcriptome.

The motifs that we found are short (8–12 bp) in comparison to known dendritic and axonal targeting elements (Andreassi and Riccio, 2009). This may be due to the possibility that our motifs are not involved in mRNA targeting but in other aspects of axonal transport like aggregation in granules. However, close examination of the targeting element in the β-actin mRNA (Zip-code), which is of 54 nucleotides, reveal a short six nucleotide sequence that is repeated two times. The position of these sequences in the secondary structure of the whole targeting element is crucial for its functionality (Ross et al., 1997; Patel et al., 2012). Therefore, it might well be, that the motifs we identified are in the core of larger elements, which are heterogeneous in their sequence. It should be noted that we did not detect a tendency for the splice sites or for the motifs to reside in the UTRS, which have been functionally defined as having localizing activity. Therefore their biological role and the rules that govern their generation remained to be determined.

Overall, our work provides the first, nucleotide level resolution transcriptome of sub cellular compartment. It is clear that although this transcriptome comprise many more mRNAs than previously estimated it is not a mirror image of the whole cell transcriptome and it contains its own unique features. It is for future studies to uncover whether there are common distinct regulatory themes for this

transcriptome in terms of its transport, translation and stability, and the role of these mRNAs in axonal biology.

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