hnRNP R and its main interactor, the noncoding RNA 7SK, coregulate the axonal transcriptome of motoneurons

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Edited by Don W. Cleveland, University of California, San Diego, La Jolla, CA, and approved February 16, 2018 (received for review December 15, 2017)

Disturbed RNA processing and subcellular transport contribute to the pathomechanisms of motoneuron diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy. RNA-binding proteins are involved in these processes, but the mechanisms by which they regulate the subcellular diversity of transcriptomes, particularly in axons, are not understood. Heterogeneous nuclear ribonucleoprotein R (hnRNP R) interacts with several proteins involved in motoneuron diseases. It is located in axons of developing motoneurons, and its depletion causes defects in axon growth. Here, we used individual nucleotide-resolution cross-linking and immunoprecipitation (iCLIP) to determine the RNA interactome of hnRNP R in motoneurons. We identified ∼3,500 RNA targets, predominantly with functions in synaptic transmission and axon guidance. Among the RNA targets identified by iCLIP, the noncoding RNA 7SK was the top interactor of hnRNP R. We detected 7SK in the nucleus and also in the cytosol of motoneurons. In axons, 7SK localized in close proximity to hnRNP R, and depletion of hnRNP R reduced axonal 7SK. Furthermore, suppression of 7SK led to defective axon growth that was accompanied by axonal transcriptome alterations similar to those caused by hnRNP R depletion. Using a series of 7SK-deletion mutants, we show that the function of 7SK in axon elongation depends on its interaction with hnRNP R but not with the PTEF-B complex involved in transcriptional regulation. These results propose a role for 7SK as an essential interactor of hnRNP R to regulate its function in axon maintenance.

Significance

Neurons are highly polarized cells. RNA-binding proteins contribute to this polarization by generating diverse subcellular transcriptomes. The RNA-binding protein hnRNP R is essential for axon growth in motoneurons. This study reports the RNA interactome for hnRNP R. The main interacting RNA of hnRNP R was the noncoding RNA 7SK. Depletion of 7SK from primary motoneurons disturbed axon growth. This effect was dependent on the interaction of 7SK with hnRNP R. Both hnRNP R and 7SK localize to axons. Loss of 7SK led to a similar depletion of axonal transcripts as loss of hnRNP R. Our data suggest that 7SK, in addition to its role in transcriptional regulation, acts in concert with hnRNP R to sort specific transcripts into axons.

Author contributions: M.B., L.S.-B., C.J., and M.S. designed research; M.B., L.S.-B., C.J., M.M., H.G., M.U., S.A., and R.B. analyzed data; and M.B., M.U., S.A., and R.B. performed research; M.U., S.A., and R.B. contributed new reagents/analytic tools; M.B., L.S.-B., C.J., M.M., H.G., M.U., S.A., and R.B. analyzed data; and M.B., L.S.-B., C.J., and M.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GEO Series accession no. GSE77101).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1721670115/-/DCSupplemental.
(Pol II) (13). More recently, the hnRNPs A1, A2/B1, R, and Q have been shown to interact with the fraction of 7SK RNA not bound to P-TEFb, thereby forming distinct 7SK/hnRNPs complexes (14–16). It has been suggested that the relative balance between 7SK/P-TEFb and 7SK/hnRNPs complexes determines the amount of free P-TEFb, which, in turn, regulates the transcriptional output of a cell (17). Nevertheless, the function of individual 7SK/hnRNkapois in general and in neuronal development in particular has remained unclear.

We show that 7SK localizes not only to the nucleus but also to the cytosol, including axons and growth cones of motoneurons, and that this localization is dependent on hnRNRP. Furthermore, we show that 7SK regulates axon growth in a manner dependent on hnRNRP. To elucidate the role of 7SK/hnRNPs complexes in axonal RNA transport, we analyzed the transcriptome of hnRNRP- and 7SK-knockdown motoneurons cultured in compartmentalized microfluidic chambers (18). As a result, we found a subset of transcripts that were up- or down-regulated similarly in axons of 7SK- and hnRNPR-knockdown motoneurons. Taken together, our results demonstrate that hnRNRP shapes the axonal transcriptome and suggest that its activity toward RNA localization is intricately linked to its main binding partner, 7SK.

**Results**

**hnRNRP Binds to the 3' UTR of mRNAs.** To understand better the mechanisms by which hnRNRP modulates axon growth in motoneurons, we used iCLIP (10) to identify RNAs interacting with hnRNRP on a genome-wide scale. We performed hnRNRP iCLIP on embryonic primary mouse motoneurons as well as on mouse NSC34 cells, which have been described previously as having motoneuron-like properties (19). For each cell type we prepared whole-cell lysates as well as one set of nuclear and cytosolic fractions (Fig. 1). Through alternative splicing of exon 2, hnRNRP gives rise to two isoforms that differ at the N terminus. Therefore, for immunoprecipitation we applied an antibody specific for the C terminus of hnRNRP (hereafter referred to as “Ab1”), which identifies both the long and the short hnRNRP isoforms, as well as an antibody (hereafter referred to as “Ab2”) specific for the N terminus of the longer isoform of hnRNRP. As negative control, we used rabbit IgG antibody for iCLIP from NSC34 cells. Compared with complete RNase digestion (+ +), partial RNase digestion (+) produced cross-linked protein–RNA complexes containing RNAs of variable size visible as a shift toward higher molecular weight (Fig. 1A). We isolated the RNA components from these complexes and processed the cross-linked RNAs for high-throughput sequencing (Table S1). A random barcode on each read was used to exclude effects from PCR amplification (10). Thus, for reads with identical barcodes mapping to the same genomic start location, only the separate barcodes are counted and quantified as cDNA counts. The nucleotide upstream of a read is defined as the cross-link site. We observed a high gene-by-gene correlation of cDNA counts between the experimental replicates for each cell type as well as between the two hnRNRP antibodies, demonstrating the reproducibility of hnRNRP iCLIP (Table S2).

After combining the datasets for each cell type (Fig. S1A), we performed sequence analysis of the hnRNRP cross-link sites. The occurrence of individual pentamers around cross-link sites was highly similar in NSC34 cells and motoneurons (Fig. 1B). The most highly enriched pentamer in both cell types was AAATT. This pentamer was also enriched when hnRNRP was immunoprecipitated with Ab2 (Fig. S1B) but not when IgG was used (Fig. S1C). Pentamer occurrence was highly correlated between individual replicates including enrichment of the AAATT pentamer, thus further demonstrating the reproducibility of hnRNRP iCLIP

![Fig. 1](image-url)

**Fig. 1.** Characterization of hnRNRP interactions with RNAs by iCLIP. (A, Upper) Autoradiogram of radiolabeled cross-linked hnRNRP R–RNA complexes following immunoprecipitation from NSC34 cells with different hnRNRP antibodies. The red rectangles depict the area on the nitrocellulose from which protein–RNA complexes following immunoprecipitation were purified for iCLIP cDNA amplification. Ab, antibody; IP, immunoprecipitation. (Lower) Western blot (WB) analysis of hnRNRP and Gapdh from the input lysates used for the immunoprecipitation. (B) Enrichment of pentamers around hnRNRP R cross-link sites in motoneurons and NSC34 cells. The Pearson correlation coefficients are shown. (C) GraphProt consensus motif at hnRNRP R cross-link sites in motoneurons. (D) Enrichment of hnRNRP R iCLIP cDNA counts in protein-coding transcripts. (E) Distribution of cDNA counts for the 3,493 hnRNRP R iCLIP targets in motoneurons. (F) GO term, Kyoto Encyclopedia of Genes and Genomes (KEGG), and InterPro analysis of motoneuron transcripts containing greater than or equal to or fewer than five hnRNRP R iCLIP cDNA counts in the combined dataset following cluster analysis.
UTRs were enriched in the cytosolic fractions at similar locations in motoneurons and hnRNP R depletion alters the somatodendritic and axonal transcriptome of motoneurons. (PNAS Latest Articles)

Within genes, cross-links were enriched in annotated 3′ UTRs (Fig. 1D). As an example, hnRNP R cross-links were present in the 3′ UTR of β-actin at similar locations in motoneurons and NSC34 cells (Fig. S2C). The zipcode region, which is normally bound by Zbp1 (21), was devoid of cross-links (Fig. S2D). When we analyzed the distribution of hnRNPR R cross-links separately for the individual experiments, we found that intronic iCLIP hits were mainly identified in the nuclear fractions, whereas interactions of hnRNPR R with 3′ UTRs were enriched in the cytosolic fractions (Fig. S2C). This indicates that nuclear hnRNPR R associates with nascent RNAs while cytoplasmic hnRNPR R specifically targets the 3′ UTR of mRNAs, as previously observed for other members of the hnRNPR family such as TDP-43 (22).

To obtain high-confidence RNA interactors, we filtered the transcripts cross-linked to hnRNPR R in motoneurons and NSC34 cells using two stringent criteria. First, we used peak finding of the combined iCLIP data to detect significant clusters of hnRNPR R-binding sites (23). Second, we considered only genes having iCLIP hits in all three replicates (combining the respective nuclear and cytosolic datasets as one replicate). As a result, we detected 3,493 transcripts in motoneurons (Dataset S1) and 2,932 transcripts in NSC34 cells (Dataset S2) as hnRNPR R targets. To obtain functional information about these transcripts, we performed gene ontology (GO) term analysis separately on transcripts with five or more or fewer than five total clustered cDNA counts (Fig. 1E). This analysis revealed an enrichment of transcripts with functions in synaptic transmission and RNA binding among those transcripts more frequently bound by hnRNPR R (Fig. 1F).

hnRNPR R modulates the Axonal Transcriptome. Given the important role of hnRNPR R in axonal β-actin mRNA translocation (3), the location of hnRNPR R cross-link sites in the 3′ UTR of mRNAs points toward a function of hnRNPR R in regulating subcellular RNA levels. To study subcellular transcriptome alterations upon loss of hnRNPR R, we virally transduced an shRNA to knock down hnRNPR R in embryonic primary mouse motoneurons cultured in microfluidic chambers (18). After 7 d in vitro (DIV) RNA was extracted from the somatodendritic and axonal compartments of four independent control and three independent hnRNPR R-knockdown motoneuron cultures. Under this condition hnRNPR R transcripts were knocked down to <50% relative to controls as measured by qPCR (Fig. 2A). To investigate transcriptome alterations in each compartment, we applied a whole-transcriptome amplification strategy followed by high-throughput sequencing (24). The knockdown of hnRNPR R transcripts measured by RNA sequencing (RNA-seq) was in concordance with the qPCR analysis (Fig. 2A). To detect transcripts altered upon hnRNPR R knockdown in the somatodendritic and axonal compartments, we performed differential-expression analysis (Fig. 2B). We found 159 transcripts up-regulated (Dataset S3) and 181 transcripts significantly down-regulated (P < 0.05) (Dataset S4) in the somatodendritic compartment of motoneurons depleted for hnRNPR R relative to controls. In the axonal compartment, the levels of 110 transcripts were increased.

Fig. 2. hnRNPR R depletion alters the somatodendritic and axonal transcriptome of motoneurons. (A) hnRNPR R transcript levels on the somatodendritic side of compartmentalized hnRNPR R-knockdown (kd) motoneurons relative to controls. Levels are shown as relative expression measured by qPCR or whole-transcriptome RNA-seq. Data represent mean ± SD. (B) Differential-expression analysis of compartmentalized hnRNPR R-knockdown motoneurons. Scatter plots show logarithmized fragments per kilobase of transcript per million mapped reads (FPKM) values as reported by cuffdiff for whole-transcriptome RNA-seq of hnRNPR R-knockdown motoneurons relative to controls. (C) Overlap of transcripts significantly (P < 0.05) deregulated in the somatodendritic and axonal compartments of hnRNPR R-knockdown motoneurons. (D) hnRNPR R binding to transcripts deregulated in hnRNPR R-knockdown motoneurons. The bar graph shows the percentage of transcripts containing at least one iCLIP hit. For the analysis grouped hnRNPR R iCLIP motoneuron data (total counts) or data from stringently filtered transcripts containing clusters of iCLIP hits as well as iCLIP hits in all three replicates (filtered counts) were used. Unchanged transcripts are defined as having a P ≥ 0.05. Numbers on the bars represent the total number of transcripts considered. ***P ≤ 0.01, ****P ≤ 0.0001; n.s., not significant; χ² test with Yates’ correction. (E) Tukey box plots showing the number of iCLIP hits per transcript. Only transcripts with at least one iCLIP hit were considered for the analysis. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001; Kruskal–Wallis ANOVA with Dunn’s multiple comparison test.

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52 transcripts were reduced upon hnRNP R knockdown (Dataset S5). Among the deregulated transcripts, 12 were up-regulated in both compartments, and seven were reduced in both compartments after hnRNP R suppression (Fig. 2C). Thus, only a minority of transcripts are altered on both the somatodendritic and axonal sides upon hnRNP R loss, while most are unique to each compartment. Nevertheless, GO term analysis revealed that transcripts with functions in translation were enriched in either compartment among up-regulated RNAs (Fig. S3A). We validated a number of axonal transcript changes by qPCR (Fig. S3B). Despite variability, which is to be expected for low-input amounts of RNA, the direction of change was in agreement with the changes predicted from the differential-expression analysis. The candidates selected for validation included the most strongly reduced protein-coding transcripts Sh2d3c and Ppia3, the transcript Apeo, which is up-regulated in ALS mice (25, 26), and the transcripts Cald1, Nes, and Pks3 with functions in axon growth (27–29).

Next, we analyzed the occurrence of iCLIP hits in transcripts that were differentially expressed in hnRNP R-knockdown motoneurons. Among axonal transcripts, 98% of the transcripts down-regulated upon hnRNP R knockdown contained iCLIP hits, compared with 79% of the up-regulated and 62% of the unchanged transcripts (Fig. 2D, total counts). The enrichment of hnRNP R binding to down-regulated transcripts was also observed when we considered only the high-confidence RNA interactors of hnRNP R (see above). In this case 51% of down-regulated transcripts contained clustered iCLIP binding sites, compared with 15% of the up-regulated and 16% of the unchanged transcripts (Fig. 2D, filtered counts). For somatodendritic transcripts, 95% of the up-regulated and 98% of the down-regulated transcripts contained iCLIP hits compared with 62% of the unchanged ones. When we considered stringently filtered iCLIP hits, 30% of the up-regulated, 36% of the down-regulated, and 16% of the unchanged transcripts contained hnRNP R-binding clusters. Likewise, when we analyzed the total number of iCLIP hits per transcript, we found that axonal transcripts down-regulated upon hnRNP R knockdown contained significantly more iCLIP hits than up-regulated or unchanged ones (Fig. 2F). In the somatodendritic compartment up- as well as down-regulated transcripts contained significantly more iCLIP hits compared with unchanged ones. Thus, hnRNP R regulates the somatodendritic as well as axonal levels of a subset of its RNA targets.

**7SK Is the Main Interacting RNA of hnRNP R.** When the hnRNP R RNA targets in motoneurons were overlapped with those in NSC34 cells, 1,481 transcripts were common to both cell types (Fig. 3A). Strikingly, the small noncoding RNA 7SK was the highest-ranked binding partner for hnRNP R in both NSC34 cells and motoneurons (Fig. 3B). The pattern of iCLIP hits along 7SK RNA was highly similar in NSC34 cells and motoneurons, showing three distinct clusters (Fig. 3C). Their positions at nucleotide resolution were virtually identical for both cell types (Fig. S4A) and were located in stem loops (SL) 1 and 3 (Fig. S4B).

Since 7SK RNA is ubiquitously expressed and highly abundant, we investigated an iCLIP dataset for the unrelated RNA-binding protein Celf4 in mouse brain (30). The fraction of cDNA counts derived from Celf4 cross-links to 7SK (transcript ENSMUSG00000065037) is much smaller than the fraction of 7SK-derived cDNA counts in our hnRNP R iCLIP motoneuron datasets (7.5 × 10⁻³ for Celf4 iCLIP vs. 8.9 × 10⁻³ for hnRNP R iCLIP). In contrast, the fractions of cDNA counts for the abundant long noncoding RNAs Malat1 and Meg3 are similar in the Celf4 and hnRNP R datasets [7.9 × 10⁻³ (Malat1) and 3.1 × 10⁻³ (Meg3) for Celf4 iCLIP vs. 4.8 × 10⁻³ (Malat1) and 5.1 × 10⁻³ (Meg3) for hnRNP R iCLIP], indicating the specificity of hnRNP R binding to 7SK. Furthermore, in NSC34
cells the fraction of hnRNP R iCLIP cDNA counts in 7SK is 2.7 × 10⁻² (Malat1: 1.7 × 10⁻²) for Ab1 and 2.5 × 10⁻² (Malat1: 2.0 × 10⁻²) for Ab2, whereas the fraction of 7SK-derived cDNA counts in the IgG iCLIP control dataset from NSC34 cells is lower, at 1.0 × 10⁻² (Malat1: 2.2 × 10⁻²).

To validate that hnRNP R and 7SK interact in vivo, we immunoprecipitated hnRNP R from NSC34 cell lysate and investigated bound RNAs by qPCR. As a result, we detected 7SK, but not Gapdh, in the immunoprecipitate (Fig. 3D). Next, we sought to verify that hnRNP R and 7SK interact directly. For this purpose we set up in vitro binding reactions containing recombinant His-tagged hnRNP R and either 7SK or its antisense RNA. We immunoprecipitated hnRNP R from these binding reactions using an anti-His antibody and found that 7SK, but not its antisense RNA, coprecipitated with hnRNP R (Fig. 3E). Thus, hnRNP R directly interacts with 7SK. Since we detected most of the head RNP R cross-links in SL1 and SL3, we generated 7SK RNAs with deletions in these regions and tested their ability to bind hnRNP R (Fig. 3F). All deletions, including either partial (ΔSL3p) or full (ΔSL3F) deletion of SL3, significantly reduced 7SK binding to hnRNP R. Taken together, these data confirm that hnRNP R binds to 7SK via interactions with SL1 and 3.

To investigate whether SL1 and 3 of 7SK also mediate binding to hnRNP R in vivo, we generated a series of knockdown constructs expressing an shRNA targeting 7SK and coexpressing the 7SK deletion mutants tested for in the in vitro binding to hnRNP R (Fig. 4A). Additionally, a construct was generated coexpressing a deletion-resistant 7SK (7SK-MM3) with mismatches in the region targeted by the shRNA. We transfected NSC34 cells with these plasmids and used the cell lysates for RNA immunoprecipitation of hnRNP R. Since transfection of cells was only partial, the cell lysate contained endogenous 7SK from untransfected cells as well as 7SK mutant RNA from transfected cells in which endogenous 7SK was knocked down. Therefore, RNA immunoprecipitation of hnRNP R allowed assessment of the binding of each 7SK mutant to hnRNP R relative to the binding of endogenous 7SK. Using this assay, we found that 7SK-MM3 RNA was able to bind to hnRNP R in vivo (Fig. 4B). In agreement with our in vitro binding assay, we observed strongly reduced binding of hnRNP R to 7SK RNAs with deletion of SL1 (ΔSL1), full deletion of SL3 (ΔSL3F), or deletions of both SL1 and 3 (ΔSL13). However, 7SK RNA with partial deletion of SL3 (ΔSL3P) retained its ability to bind to hnRNP R under these conditions. Thus, the interaction of hnRNP R with 7SK in vivo also requires SL1 and SL3.

**hnRNP R Regulates the Subcellular Localization of 7SK.** Even though 7SK is an abundant nuclear RNA, we found cross-linked 7SK/hnRNP R complexes enriched in the cytosolic fractions of both motoneurons and NSC34 cells (Fig. 5A). These 7SK/hnRNP R complexes could be immunoprecipitated with both Ab1 and Ab2. In contrast, interactions with the abundant long noncoding RNAs Malat1 and Xist were mainly observed in the nuclear fractions, corresponding to their known nuclear localization. This result suggests that 7SK/hnRNP R complexes translocate from the nucleus to the cytosol. In a previous study using compartmentalized motoneurons, we detected 7SK in axons of cultured motoneurons (24). Using in situ hybridization, we found that the cytosolic localization of 7SK extends not only into axons but even into the growth cones of motoneurons (Fig. 5B). In axons, we observed 7SK-positive punctae in close proximity to hnRNP R-labeled structures (Fig. 5C and D). Thus, while being located mainly in the nucleus, both 7SK and hnRNP R localize to axons of developing motoneurons.

Since 7SK is the main RNA target for hnRNP R, we examined a putative role for hnRNP R in regulating the cytosolic localization of 7SK by using lentiviral knockdown of hnRNP R in motoneurons. After 5 DIV hnRNP R protein levels were reduced by ∼90% for the long isoform and ∼70% for the short isoform (Fig. 5E). hnRNP R RNA levels were similarly reduced, while total 7SK levels were unaffected by hnRNP R knockdown (Fig. 5F). Quantification of 7SK levels in the nuclei of hnRNP R-knockdown motoneurons revealed no significant alterations (Fig. 5G), whereas 7SK-positive punctae almost completely disappeared from axons upon loss of hnRNP R (Fig. 5H). Thus, hnRNP R depletion does not alter nuclear 7SK levels but selectively reduces axonal levels of 7SK RNA in motoneurons.

7SK Regulates Axon Growth in an hnRNP R-Dependent Manner. hnRNP R has previously been implicated in axon growth and guidance (3).
Since 7SK is the most prominent RNA target for hnRNP R, we investigated the effect of 7SK RNA depletion on axon growth. For this purpose we knocked down 7SK by lentiviral shRNA transduction and cultured the knockdown motoneurons until 7 DIV, a time point at which axons have reached a length often exceeding 500 μm. While 7SK RNA was substantially reduced in knockdown motoneurons (Fig. 6A), their survival was not affected (Fig. 6B). Compared with control axons reaching a median length of >400 μm after 7 DIV, 7SK knockdown motoneurons had shorter axons with a median length of ∼300 μm (Fig. 6C and D). Thus, the observation that 7SK knockdown interferes with axon elongation but not with the survival of motoneurons in vitro is similar to the phenotype of hnRNP R depletion.

Next, we investigated whether the interaction between 7SK and hnRNP R is necessary for the role of 7SK in axon elongation. For this purpose we assessed the ability of the different 7SK mutant RNAs (Fig. 3F) to rescue axon growth after depletion of endogenous 7SK. We generated lentiviruses from the constructs expressing an shRNA against 7SK and coexpressing mutant 7SK (Fig. 4A) and transduced primary motoneurons to measure their axon lengths after 7 DIV. We found that wild-type 7SK, 7SK-MM3, and 7SK-ΔSL3P were able to restore axon extension in 7SK-knockdown motoneurons, while 7SK-ΔSL3F and 7SK-ΔSL1 failed to rescue this effect (Fig. 6E). Since 7SK-ΔSL3P but neither 7SK-ΔSL3F nor 7SK-ΔSL1 can bind to hnRNP R in vivo, this result suggests that 7SK regulates axon growth through hnRNP R binding.

An important function of 7SK is to regulate transcription by binding to the P-TEFb complex composed of CDK9 and cyclin T1. We used an RNA pulldown assay to test our 7SK mutant RNAs for their ability to bind to P-TEFb (Fig. 7A). We hybridized a biotinylated antisense oligonucleotide to 7SK wild-type or mutant RNAs and incubated this duplex with NSC34 lysate. Following immobilization to streptavidin beads, we investigated bound P-TEFb components by immunoblotting. The antisense oligonucleotide was able to bind to and pull down all 7SK mutants (Fig. 7B). Following incubation with cell lysate, wild-type 7SK was bound to Cdk9 and cyclin T1 as well as to Larp7, which is essential for 7SK stability (Fig. 5).

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7C) (31). As a negative control, 7SK antisense RNA purified with a biotinylated sense oligonucleotide did not copurify any of these components. Among the mutant 7SK RNAs, those mutants with deletion of SL1 (ΔSL1 and ΔSL13) failed to pull down Cdk9 or cyclin T1 (Fig. 7C). In contrast, mutants with deletions in SL3 (ΔSL3P and ΔSL3F) were able to bind to the P-TEFb components. This is in agreement with a previous report showing that P-TEFb is recruited to 7SK through SL1 and SL4 (32). Importantly, all mutants bound Larp7, indicating that they are otherwise stable. Taken together, our results suggest that the interaction of 7SK with hnRNP R is necessary for axon growth, since the 7SK mutant ΔSL3F is unable to rescue the axonal defect of 7SK-deficient motoneurons even though it is capable of binding to P-TEFb.

**A Subset of Axonal Transcripts Is Similarly Altered After Knockdown of 7SK or hnRNP R.** Our data point toward the possibility that hnRNP R and 7SK act in concert to regulate the subcellular transcriptome of motoneurons. Thus, we investigated subcellular transcriptome alterations in compartmentalized 7SK-knockdown motoneurons. For this purpose we extracted somatodendritic and axonal RNA from four independent 7SK-knockdown motoneuron cultures and performed whole-transcriptome profiling as before. 7SK levels were suppressed by ~50% as measured by qPCR (Fig. 8A). Since our whole-transcriptome amplification method also captures nonpolyadenylated transcripts, including 7SK, we were able to detect its depletion by ~50% in the RNA-seq data as well (Fig. 8A). Transcripts altered upon 7SK knockdown relative to controls were detected in the somatodendritic and axonal compartments by differential-expression analysis (Fig. 8B). In the somatodendritic compartment 162 transcripts were up-regulated (Dataset S7) compared with 137 transcripts that were down-regulated significantly (P < 0.05) (Dataset S8). In the axonal compartment 137 transcripts were up-regulated (Dataset S9), and 46 transcripts were down-regulated (Dataset S10).

Since 7SK has a function in transcriptional regulation by inactivating P-TEFb, we investigated the binding of NELF, DSIF, and Pol II to the promoter regions of the genes that were deregulated in the somatodendritic compartment of 7SK-knockdown motoneurons. For this purpose we analyzed ChIP-seq data for NELF, DSIF, and Pol II to quantify their interaction with the promoter regions of
these genes (33). We found that transcripts up-regulated upon 7SK knockdown contain significantly more ChIP-seq reads for NELF, DSIF, and Pol II from ChIP-seq data around the transcription start site (TSS) of transcripts significantly deregulated in the somatodendritic compartment of 7SK-knockdown motoneurons relative to controls. The ChIP-seq data were derived from Liu et al. (33). ****P ≤ 0.0001; Mann–Whitney test. (D) Overlap of transcripts significantly (P < 0.05) deregulated in the somatodendritic and axonal compartments of 7SK-knockdown motoneurons. (E) Scatter plot showing logarithmized transcript fold change of axonal transcripts significantly (P < 0.05) deregulated in 7SK and hnRNP R-knockdown motoneurons. Pearson’s r is shown. (F) List of transcripts significantly down-regulated in axons of hnRNP R-knockdown and 7SK-knockdown motoneurons. Included are the number of cDNA counts from the grouped motoneuron hnRNP R iCLIP dataset and the cDNA counts of TDP-43 iCLIP from human brain samples. kd, knockdown. (G) Model of 7SK/hnRNP R complex functions in motoneurons.
Fig. S5. Nes NTR, which points to—and other transcripts | (25). In contrast, among the PNAS Latest Articles
results provide evidence that RNA-binding proteins can act to-| together with noncoding RNAs to regulate the subcellular abun-
dance of transcripts underlying important cellular processes such as the differentiation and growth of axons.

The interactions identified by iCLIP suggest that hnRNPR binds to a large number of transcripts in motoneurons, many of which are associated with axon guidance and synapse functions. The binding sites of hnRNPR were enriched in the 3′ UTR, which points toward a role for hnRNPR in transcript subcellular transport and/or stability. Using compartmentalized motoneuron cultures combined with whole-transcriptome profiling, we found that loss of hnRNPR led to distinct transcriptome alterations in axons, including down-regulation of transcripts encoding proteins with cytoskeletal functions such as Cald1, Nes, and Pls3. Interestingly, PL33 (plastin 3), an actin-bundling protein, has previously been identified as a modifier in SMA (29). Among down-regulated transcripts was Apoe, which is increased in the spinal cords of patients with ALS (25). Apoe overexpression causes cytoskeletal instability and disrupts axonal transport (35, 36). Based on these findings, future experiments must show whether the cytoskeletal integrity is disrupted in axons of hnRNPR-deficient motoneurons. Importantly, down-regulated but not up-regulated axonal transcripts were associated with significantly more hnRNPR iCLIP hits compared with unregulated transcripts. This suggests that the axonal transcripts down-regulated upon hnRNPR knockdown are those actively transported by hnRNPR, whereas those up-regulated, including many components of the translational machinery, might represent compensatory mechanisms. Additionally, the set of transcripts deregulated in the axonal compartment of hnRNPR-knockdown motoneurons was largely distinct from the transcript changes in the somato-dendritic compartment. Thus, transcript alterations in the axons of knockdown motoneurons are not simply due to alterations in their abundance in the cell body but might rather reflect an active mechanism toward their transport or stability in axons.

When we evaluated the transcripts bound by hnRNPR in both NSC34 cells and motoneurons, the short noncoding RNA 7SK was the most highly enriched target. This is in agreement with previous reports identifying hnRNPR as a component of 7SK particles (14–16). Our iCLIP data add to this knowledge by showing that hnRNPR directly interacts with 7SK in vivo and that this interaction occurs within SL1 and SL3 of 7SK. We confirmed this interaction using an in vitro assay containing purified hnRNPR and different 7SK mutant RNAs. Additionally, we investigated hnRNPR binding to 7SK in vitro by RNA immunoprecipitation. Interestingly, while partial deletion of SL3 (ΔSL3P) abolished 7SK binding to hnRNPR in vitro, binding to hnRNPR in a cellular context was largely unaffected by this deletion. This suggests that hnRNPR recruitment to 7SK in cells might be supported by additional 7SK-interacting proteins. Nevertheless, complete removal of SL1 or SL3 abolished binding of hnRNPR R both in vitro and in vivo, thereby confirming the functional relevance of the hnRNPR R cross-linking sites in these regions.

Given that 7SK is the main interactor of hnRNPR, an important question is whether loss of 7SK similarly leads to reduced axon growth and whether such a defect depends on its interaction with hnRNPR. Indeed, we found that knockdown of 7SK in motoneu-rons led to reduced axon growth which resembles the axon growth defect following hnRNPR depletion. Furthermore, we were able to rescue this defect by coexpressing knockdown-resistant 7SK, which confirms the specificity of this phenotype. Importantly, a rescue effect was also observed by coexpression of the 7SK ΔSL3P but not of the ΔSL3F deletion mutant. Since ΔSL3P but not ΔSL3F can bind hnRNPR R in vivo, this further indicates that the role of 7SK in axon elongation is mediated through 7SK/hnRNPR R complexes. However, we cannot rule out the possibility that additional RNA-binding proteins interact with SL1 and/or SL3 and thereby regulate the functional role of 7SK in axon growth. Future experiments investigating the protein interactionome of 7SK/hnRNPR complexes in motoneurons will help resolve this question. Furthermore, while the 7SK mutant ASL3F did not bind hnRNPR R, it was still able to recruit the P-TEFb complex. Given that 7SK ΔSL3F was unable to rescue the axonal defect of 7SK-knockdown motoneurons, this result points toward the possibility that a transcription-independent function of 7SK/hnRNPR R complexes mediates this effect.

To further test the hypothesis that 7SK serves additional functions beyond modulating transcription (such as in regulating translocation of specific transcripts into the axon of motoneurons), we investigated transcriptome changes in compartmentalized 7SK-knockdown motoneuron cultures and compared them with those obtained after hnRNPR R knockdown. This showed that a subset of axonal transcripts is regulated in a similar manner by hnRNPR R and 7SK. Importantly, these transcripts also harbor hnRNPR R cross-linking sites, which indicates that their axonal reduction in hnRNPR R-knockdown motoneurons might be a direct consequence of hnRNPR R deficiency and disassembly of 7SK/hnRNPR R complexes. The down-regulated transcripts encode proteins known to play a role in axon growth and cytoskeleton assembly, suggesting a reason for the disturbed axon-elongation phenotype of motoneu-
rons depleted of hnRNPR R. One of these candidates, Gmpthb, has recently been implicated in axon growth and guidance. Gmpthb is a glycoprotein located in neurites, and Gmpthb-deficient neurons exhibit shorter axons in vitro and defective axon guidance in vivo (34, 37). Thus, the combined loss of Gmpthb and other transcripts encoding proteins with cytoskeletal functions, such as Cald1, might negatively affect axon growth. In contrast, several up-regulated transcripts encode ribosomal proteins. This finding is of interest, considering that an increased number of axonal ribosomes has been detected in a mouse model of ALS (38). Increased numbers of ribosomes might reflect a mechanism whereby an enhanced capacity for protein synthesis compensates for the reduced availability of functionally relevant transcripts. At the same time, up-regulation of specific ribosomal proteins might also have the opposite effect and disrupt ribosomes due to alterations of their stoichiometry (39). Future experiments directed toward measuring the rate of protein synthesis in hnRNPR R- and 7SK-deficient motoneurons could help further clarify this point.

Interestingly, we also detected cross-linked 7SK/hnRNPR R complexes in the cytosolic fractions of motoneurons, which indicates either that this complex that has originally been identified in the nucleus translocates to the cytosol or that 7SK and hnRNPR R are
assembled into mesosomal ribonucleoprotein (mRNP) complexes once they have been transported independently to the cytosol. Since hnRNP R has been found to interact with a number of proteins linked to ALS, including TDP-43, it is tempting to speculate that transport mRNPs composed of 7SK/hnRNP R and other hnRNPs act in a cooperative manner to transport RNA's into axons or regulate their subcellular stability in axons (Fig. S6). In agreement, we detected TDP-43 cross-links on those RNA's down-regulated in axons of hnRNP R- and 7SK-deficient motoneurons. Future studies directed toward identifying the protein and RNA composition of these particles will help unravel the mode of action of hnRNP R toward subcellular RNA localization.

Taken together, the close interaction of hnRNP R with 7SK and the similar repertoire of transcripts that are deregulated in axons of knockdown motoneurons indicate that 7SK and hnRNP R act together in the assembly and sorting of mRNP complexes for transcripts that are locally translated in axons to serve an essential role in axon growth.

Materials and Methods

Animals. CD-1 mice were housed in the animal facilities of the Institute of Clinical Neurobiology and Neuroprotection at the University Hospital of Wuerzburg. Mice were maintained in a 12-h/12-h day-night cycle under controlled conditions at 20–22 °C and 55–65% humidity with food and water in abundant supply. Experiments were performed strictly following the regulations on animal protection of the German federal law and of the Association for Assessment and Accreditation of Laboratory Animal Care, in agreement with and under control of the local veterinary authority and Committee on the Ethics of Animal Experiments (Regierung von Unterfranken).

Primary Mouse Motoneuron Culture. Motoneurons were prepared from E12.5 CD-1 mouse embryos as previously described (40). They were then cultured in microfluidic chambers using a BDNF gradient according to a published protocol (18).

iCLIP. iCLIP was performed as described previously (10). For subcellular fractionation, cells were lysed in fractionation buffer [50 mM Tris HCl (pH 7.4), 100 mM NaCl, 0.1% Nonidet P-40].

RNA-Seq. For RNA-seq of compartmentalized motoneurons, we used a whole-transcriptome profiling method described previously (24).

RNA Immunoprecipitation. NSC34 cells were lysed [20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgO2, 2 mM DTT, 1% Nonidet P-40 buffer] followed by immunoprecipitation with anti-hnRNP R antibody (Abcam) or control rabbit IgG (Santa Cruz Biotechnology). Bound RNA's were extracted with TRIzol (Thermo Fisher), reverse-transcribed, and quantified by qPCR.

In Vitro Binding Assay. Protein G (Thermo Fisher) beads bound to anti-His antibody (Abgent) or control mouse IgG (Santa Cruz Biotechnology) were incubated with recombinant His-hnRNP R and 7SK RNA's generated by in vitro transcription in lysis buffer. Captured RNA was purified by proteinase K digestion and phenol-chloroform extraction, reverse-transcribed, and analyzed by qPCR.

RNA Pulldown Assay. 7SK RNA's generated by in vitro transcription were captured on Pierce streptavidin beads (Thermo Fisher) with a biotinylated antibody and incubated with NSC34 cell lysate. Bound protein's were analyzed by SDS/PAGE and Western blotting.

A detailed description of the experimental and data analysis procedures can be found in SI Materials and Methods.

Acknowledgments. We thank Crt Gorup and Tomaz Curk for bioinformatics support and Carsten Ade, Wolfgang Haidelt, and Victoria McParland for high-throughput sequencing. This work was supported by Deutsche Forschungsgemeinschaft program SP1738 [Grants BR49101-1 (to M.B.), SE697/4-1 (to M.S.), and BA2168/11-1 (to R.B. and M.U.)] and program SPP1935 [Grants BR49102-1 (to M.B.) and SE697/5-1 (to M.S.).]