Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits

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Edited* by Louis M. Kunkel, Children's Hospital Boston, Boston, MA, and approved May 12, 2011 (received for review March 29, 2011)

Spinal muscular atrophy (SMA), caused by the deletion of the *SMN1* gene, is the leading genetic cause of infant mortality. SMN protein is present at high levels in both axons and growth cones, and loss of its function disrupts axonal extension and pathfinding. SMN is known to associate with the RNA-binding protein hnRNP-R, and together they are responsible for the transport and/or local translation of β-actin mRNA in the growth cones of motor neurons. However, the full complement of SMN-interacting proteins in neurons remains unknown. Here we used mass spectrometry to identify HuD as a novel neuronal SMN-interacting partner. HuD is a neuron-specific RNA-binding protein that interacts with mRNAs, including candidate plasticity-related gene 15 (cpg15). We show that SMN and HuD form a complex in spinal motor axons, and that both interact with cpg15 mRNA in neurons. CPG15 is highly expressed in the developing ventral spinal cord and can promote motor axon branching and neuromuscular synapse formation, suggesting a crucial role in the development of motor axons and neuromuscular junctions. Cpg15 mRNA previously has been shown to localize into axonal processes. Here we show that SMN deficiency reduces cpg15 mRNA levels in neurons, and, more importantly, cpg15 overexpression partially rescues the SMN-deficiency phenotype in zebrafish. Our results provide insight into the function of SMN protein in axons and also identify potential targets for the study of mechanisms that lead to the SMA pathology and related neuromuscular diseases.

neuritin | embryonic lethal abnormal vision Drosophila-like 4 (ELAV-L4) | local protein synthesis

Spinal muscular atrophy (SMA) is a devastating genetic disease leading to infant mortality, due mainly to the loss of α-motor neurons of the spinal cord and brainstem nuclei. SMA occurs due to depletion of a ubiquitously expressed protein, SMN, which in all cells regulates RNA biogenesis and splicing through its role in the assembly of small nuclear ribonucleoprotein (snRNP) complexes (1). Despite the well-characterized association of SMN with the snRNP complex in both the nucleus and cytoplasm of motor neurons, in the axons SMN associates with mobile ribonucleoprotein (RNP) particles that are free of the core snRNP complex proteins (2). Thus, it is hypothesized that SMN may function in the assembly of axonal RNPs to regulate axonal mRNA transport and/or local protein synthesis (3, 4). Deficits in mRNA transport and local mRNA translation are associated with such neurologic disorders as fragile X syndrome and tuberous sclerosis (5, 6). Therefore, the interaction of SMN complex with other RNPs and their associated mRNAs within the axon may be crucial to understanding the pathophysiology of SMA.

At present, the only RNP known to bind SMN in the axons is hnRNP-R, which regulates β-actin mRNA localization in growth cones (4). In fact, dissociated motor neurons from a severe SMN-deficiency mouse model, *Smn*$^{-/-}$*;SMN2tg*, display defects in axonal growth and growth cone morphology and contain reduced levels of β-actin mRNA in growth cones (4), lending credence to the hypothesis that SMN complex functions in mRNA transport, stability, or translational control in the axons. Reduced SMN levels may disrupt the assembly of such RNPs on target mRNAs, leading to decreased mRNA stability and possibly transport within the axons. Here we identify HuD as a novel interacting partner of SMN, and show that SMN and HuD both colocalize in motor neurons and bind to the candidate plasticity-related gene 15 (cpg15) mRNA. We also demonstrate that cpg15 mRNA colocalizes with SMN protein in axons and is locally translated in growth cones. Finally, we show that in zebrafish, cpg15 overexpression partially rescues SMN deficiency, demonstrating that cpg15 may serve as a modifier of SMA deficiency. These results identify cpg15 as an mRNA target for the SMN–HuD complex and provide potential avenues for studying the mechanisms of SMN function in motor neuron axons.

Results

To examine the functional interactions of SMN in developing neurons, we performed mass spectrometry (MS) analysis of proteins that coimmunoprecipitate with the SMN complex. Unlike previous studies that investigated interactions of SMN using cell lines that overexpressed SMN with epitope tagging (7), we used primary embryonic cortical neurons to immunoprecipitate the endogenous SMN complex. We performed coimmunoprecipitation (co-IP) experiments under high stringency conditions to isolate proteins that associate with SMN, and chose a 1% false discovery rate (FDR) cutoff for our database searches to ensure minimal false-positive peptide hits. As predicted from the previous establishment of SMN's involvement in RNA biogenesis and splicing, the MS data from neuronal SMN immunoprecipitation (IP) showed a strong association with known SMN interaction partners, including Gemin 2–8, RNA helicases (Ddx3x and Ddx17), nucleolin (1, 7, 8), and hnRNP-R (9) (Fig. 1*A* and Table S1). In addition, we detected a novel interaction with the RNA-binding protein (RBP) HuD (also known as ELAV-L4), with a consistently low FDR (Fig. 1 *A* and *B*). HuD is a neuron-specific RBP of the Hu/Elav-family that regulates the stability of mRNAs important for neural development and plasticity (10, 11).

To further confirm the interaction of SMN with HuD, we performed reciprocal IPs of the endogenous proteins that were

Author contributions: B.A., J.H., C.E.B., J.A.J.S., and M.S. designed research; B.A., M.J.K., L.T.H., M.H.W., S.S., and D.N. performed research; T.T.M. and J.L.T. contributed new reagents/analytic tools; B.A., M.J.K., L.T.H., M.H.W., D.N., C.E.B., J.A.J.S., and M.S. analyzed data; and B.A., C.E.B., J.A.J.S., and M.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1104928108/-/DCSupplemental.

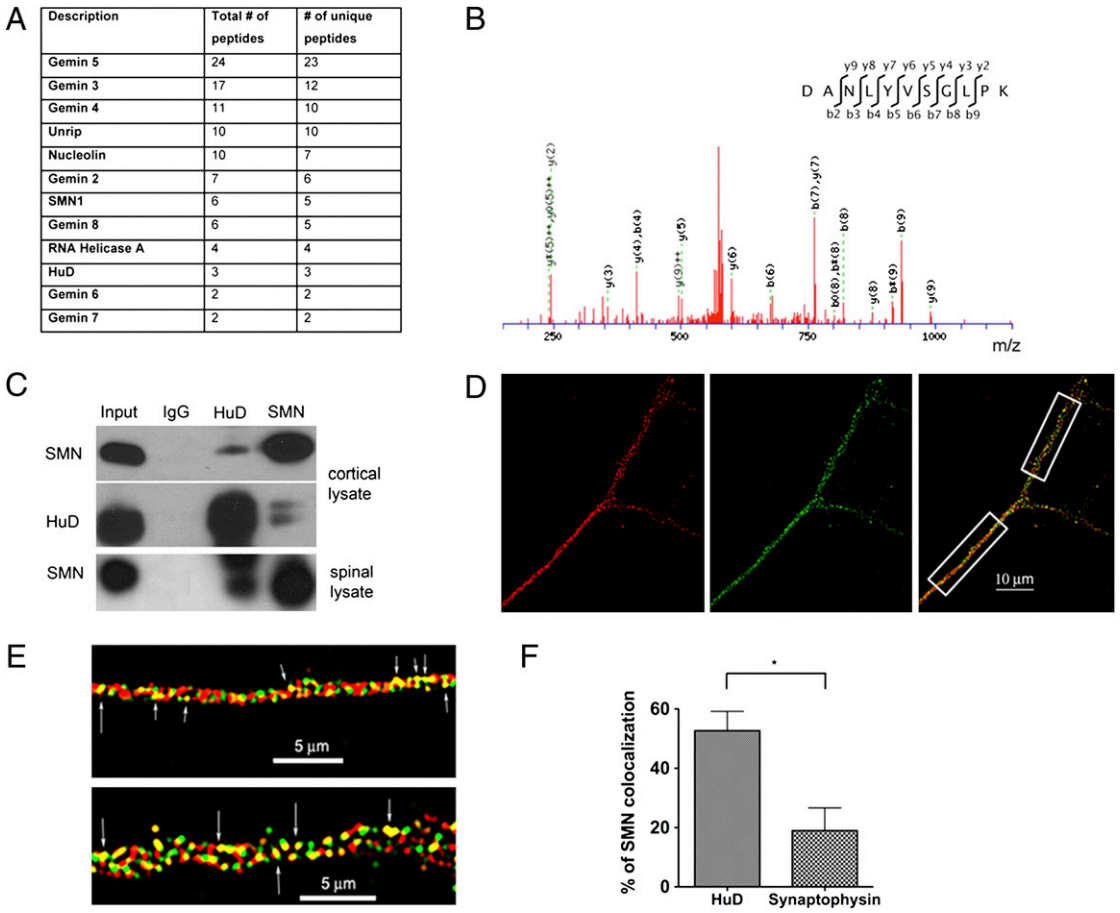

Fig. 1. SMN interacts with HuD. (*A*) List of proteins that coimmunoprecipitate with SMN from cortical neurons with an FDR ~1%. All peptides with a Mascot score >35 are listed. All proteins listed were found in three biological replicates. Unique peptides observed for each protein are listed in Table S1. (*B*) Mass spectrum of a peptide derived from HuD identified in SMN IP from cortical neurons. HuD protein is a strong interactor, with a protein score of 75 and an FDR of ≤1%. (*C*) IP from spinal cord lysates using the SMN, HuD, or IgG antibody. A 38-kDa band representing SMN protein is observed in the HuD IPs, and a 40- to 45-kDa band specific for HuD is seen in the SMN IPs. (*D*) Immunofluorescence with anti-SMN (green) and anti-HuD (red) showing colocalization of the HuD protein with SMN granules along the motor neuron axon (0.1-µm section). (*E*) Detail of *D* showing colocalization of SMN with HuD. Arrows indicate fully overlapping signals. (*F*) Graph of the mean ± SEM percentage of SMN granules that colocalize with the HuD signal or synaptophysin signal ($P < 0.005$, Student *t* test; $n = 7–10$ per group).

identified in the co-IP from embryonic neuronal tissues. In both cortical and spinal cord lysates, we detected endogenous HuD and SMN proteins in Western blot analyses of SMN IPs and HuD IPs, respectively (Fig. 1*C*). We then quantitatively analyzed the colocalization of SMN and HuD proteins in 5 d in vitro (DIV) primary motor neuron cultures that were derived from E13 mouse spinal cord by immunopanning (Figs. S1*A* and S2). Motor neurons that were double-labeled with anti-SMN and anti-HuD or anti-synaptophysin antibodies were quantified for colocalization. Quantitative analyses demonstrated a statistically nonrandom colocalization of SMN with HuD within the granules localizing to neuronal processes, with a mean value of 52.7% ± 6.5%, where the signal for SMN protein fully overlapped with the signal for HuD protein (Fig. 1 *D–F*). In contrast, the colocalization of SMN with synaptophysin, a relatively abundant protein in the axons (2), only revealed 19.0 ± 7.7% overlap (Fig. 1*D* and Fig. S1*B*). This overlap of SMN with synaptophysin differed significantly from the colocalization of SMN with HuD (Fig. 1*F*; $P \leq 0.005$) and was similar to that previously reported for axonal SMN colocalizing with Gemin proteins (2). Together with our co-IP data, these results indicate that SMN associates with HuD in motor neurons, and that these two proteins colocalize in granules within motor neuron axons.

HuD stabilizes mRNAs by binding to AU-rich elements (AREs) located in their 3′ UTR sequences (11, 12), and may promote translational initiation (13). We reasoned that in developing neurons, the interaction between HuD and SMN might regulate the stability and/or transport of a subset of mRNAs; thus, we focused on mRNAs that are essential during neuronal growth and differentiation, including those identified as HuD targets (GAP43, cpg15, tau, and Homer) (14–17). Among the mRNAs that we tested, cpg15 mRNA demonstrated relatively high abundance in HuD IPs compared with other mRNAs (Fig. 2*A* and Table S2). Although there is no evidence suggesting that mRNAs bind SMN directly, such mRNAs as β-actin have been shown to bind and colocalize with the SMN-binding partner hnRNP-R along the axons (4, 18, 19). Similarly, we examined whether mRNAs abundant in HuD IPs could be detected in SMN IPs as well, and found that levels of the mRNAs cpg15, β-actin, and GAP43 were comparably detectable in SMN IPs (Fig. 2*B*). Because cpg15 mRNA was the most abundant mRNA in HuD IPs and was also detected in SMN IPs, we decided to analyze whether this mRNA may play a role in defects induced by SMN deficiency in primary neurons.

Cpg15 (also known as neuritin), is expressed in postmitotic neurons, and its expression can be induced by neuronal activity and growth factors (20). CPG15 protein is known to protect cortical neurons from apoptosis by preventing activation of the caspase pathway (21). In *Xenopus*, CPG15 protein is expressed in the developing ventral spinal cord (22) and is involved in motor

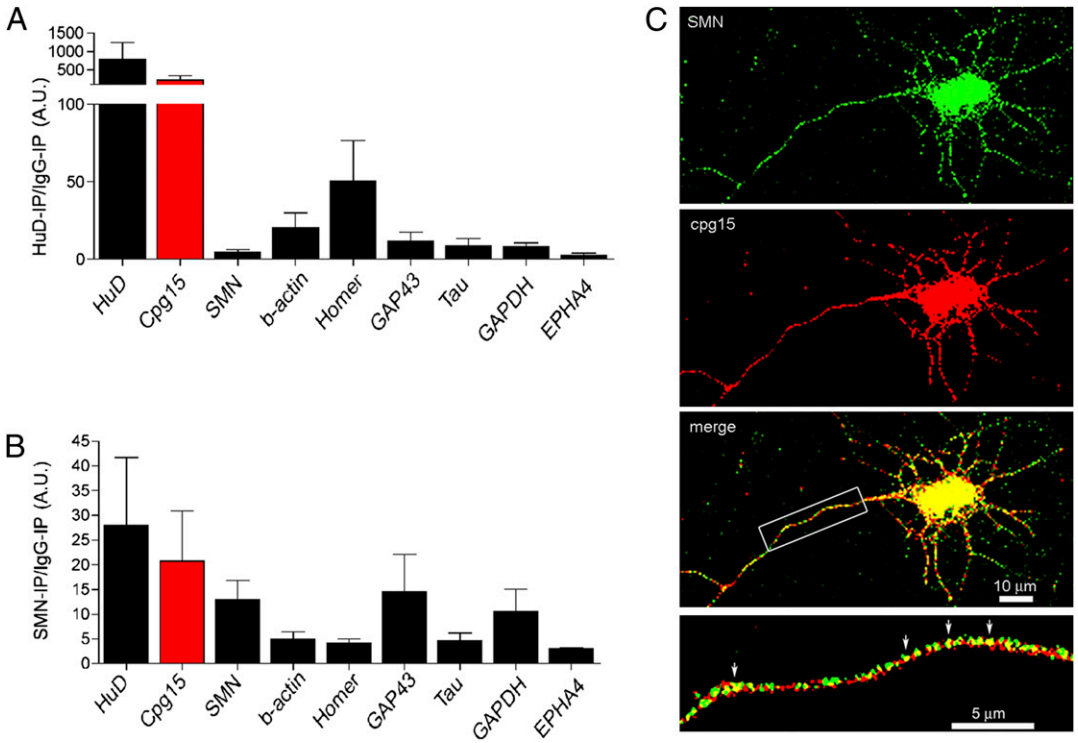

Fig. 2. Cpg15 mRNA interacts with HuD and SMN in vitro and in vivo. RNA was isolated from the HuD-IP (*A*) and SMN-IP (*B*) complexes of cortical neurons. RT-PCR was performed using primers specific for HuD, SMN, cpg15, β-actin, Homer, GAP43, Tau, EphA4, and GAPDH ($n = 6$, from three independent experiments and two technical replicates; Table S2). Data represent values normalized to IgG-IPs. (*C*) cpg15 mRNA (red) colocalizes with SMN protein (green) in motor neuron axons. (*Inset*) Arrows show colocalization of SMN and cpg15 mRNA within the axon.

neuron axon branching and neuromuscular synapse formation (23), suggesting that it plays a role in the development of motor neuron axons and neuromuscular synapses. Interestingly, cpg15 mRNA contains several AREs in its 3′ UTR that are known to interact with HuD protein (14, 17, 24) and is present in axons of cultured DRG neurons (16). Taking these previous observations into account, we analyzed cpg15 mRNA expression and colocalization with SMN in embryonic mouse motor neuron axons. We observed punctate staining for cpg15 mRNA that colocalized with SMN protein along the motor neuron axons (Fig. 2*C*). A negative control cpg15 scrambled probe displayed faint background staining (Fig S3). Quantitative analyses of cpg15 punctae that colocalized with SMN demonstrated a statistically nonrandom colocalization, with a mean value of 37.4% ± 4.5% (mean ± SEM; $n = 6$). This pattern of colocalization suggests that SMN may play a regulatory role in the local stability and/or translation of cpg15 mRNA within the motor neuron axon and growth cone.

Our finding that SMN protein associates with HuD protein and the HuD target cpg15 mRNA in neurons led us to ask whether SMN deficiency affects the abundance or cellular distribution of cpg15 mRNA. To address this question, we compared the amount of cpg15 mRNA in the neurite and cell body compartments of SMN knockdown and control neurons. We used the modified Boyden chamber system to harvest neurite-enriched lysates from cortical neuronal cultures (6) in which cells were infected with lentivirus containing either SMN or control shRNAs (Fig. S4). In neurons with reduced SMN protein levels, cpg15 mRNA was substantially diminished in both the cell body and neurite compartments compared with control cultures (Fig. 3*A*). Other axonal mRNAs, including β-actin and GAPDH (25), did not show a significant change with SMN knockdown, suggesting that only selected mRNAs are affected in our experimental model (Fig. 3*A*). These results indicate that SMN deficiency affects cpg15 mRNA levels.

Given the colocalization of SMN protein with HuD protein and cpg15 mRNA in axons, we wanted to know whether cpg15 mRNA is transported to and locally translated within the axons. To explore this, we used a reporter construct encoding a modified GFP transcript fused to the full-length 3′ UTR of cpg15. The GFP protein encoded by this construct is myristoylated and destabilized (dGFP^{myr}-cpg15 3′ UTR), resulting in its restricted mobility and rapid turnover, respectively. Expression of this plasmid in hippocampal neurons at 2 DIV resulted in a robust GFP signal in cell bodies, axons, and axon terminals (Fig S5). Using the fluorescence recovery after photobleaching (FRAP) method, we photobleached transfected hippocampal neurons at the distal axonal terminal (i.e., five or six cell body lengths away from the soma, ~50–60 μm) including the growth cone, and then monitored these neurons for signal recovery over a period of ~12 min (Fig. 3 *B* and *C*). We used the translational inhibitor cycloheximide (CHX) as a control for translational recovery. We observed that the dGFP^{myr}-cpg15 3′ UTR fluorescence signal recovery in control axons was rapid after bleaching, whereas the dGFP^{myr}-cpg15 3′ UTR fluorescence recovery in axons pretreated with CHX was significantly repressed, indicating that the observed recovery is indeed translation-dependent (Fig. 3 *B* and *C*). Taken together, these results indicate that the 3′ UTR of cpg15 mRNA is sufficient for its transport along axons and localized translation within the axons.

Because previous studies have shown that CPG15 is important for motor neuron axonal growth and synaptogenesis in *Xenopus* (23), we asked whether CPG15 overexpression could modify SMA phenotype in vivo. To date, *SMN2* and *plastin 3* are the only two identified genes that act as modifiers of SMA pathology (26, 27). To investigate whether *cpg15* might serve as a modifier gene of SMN deficiency, we took advantage of the well-characterized zebrafish *smn*-morpholino (MO) model, in which motor axon development is specifically perturbed when SMN levels are decreased

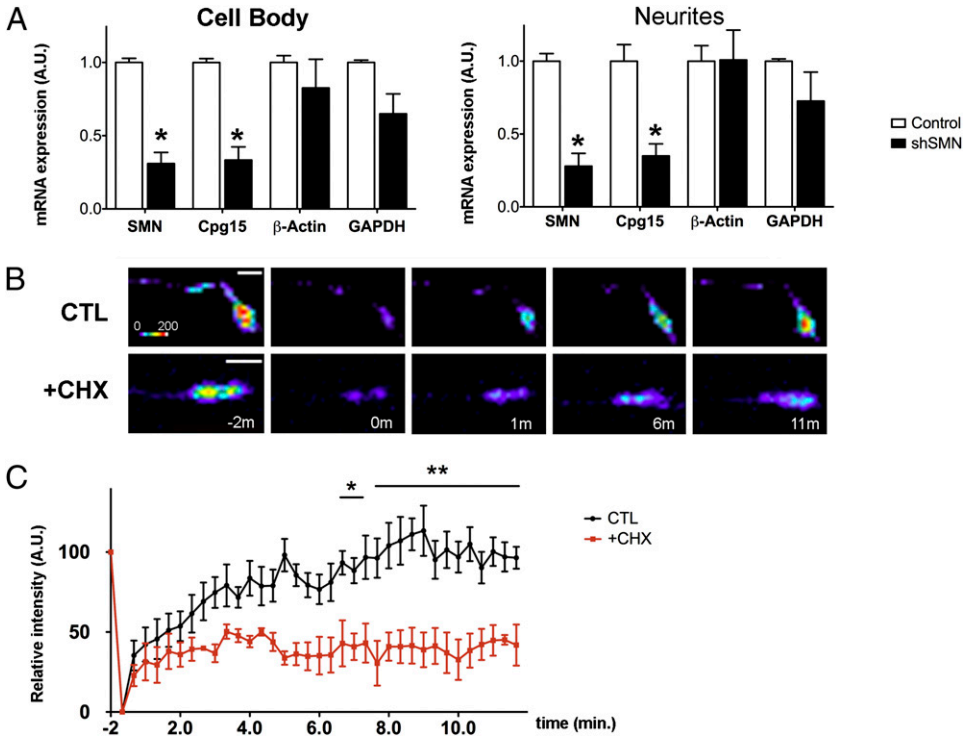

Fig. 3. Cpg15 mRNA distribution and local translation. (*A*) cpg15 mRNA expression decreases in the neurites when SMN levels are reduced. Cortical neurons were infected with lentivirus carrying either SMN shRNA (shSMN) or the control empty vector (CTL). RNA was isolated from both compartments of the Boyden chamber after 10DIV. Equal amounts of RNA was used to prepare cDNAs and quantitative real-time RT-PCR was performed using primers specific for SMN, cpg15, β-actin, and GAPDH mRNA. Results are normalized to control conditions for the cell body and the neurites separately. $*P < 0.0001$, Student *t* test; $n = 12$, from six independent biological sets. Data are presented as mean ± SEM. (*B*) cpg15 is locally translated in the growth cones. Representative images demonstrate the fluorescence recovery after photobleaching (FRAP) in distal hippocampal axons transfected with $dGFP^{myr}$-cpg15 over a period of 12 min in the absence (*Upper*) or presence (*Lower*) of 100 μM CHX. (Scale bar: 10 μm.) (*C*) Quantification of GFP intensity in the distal growth cones during prebleaching and postbleaching of hippocampal neurons in the absence (CTL, black line) or presence (+CHX, red line) of CHX. For each time point, the data represent an average percentage of prebleach intensity ± SEM, with the prebleach intensity normalized to 100 ($n = 3$ for the +CHX group; $n = 5$ for the CTL group). Significant recovery was observed by two-way ANOVA when comparing the CTL and +CHX groups at each time point: $*P < 0.05$ from 6.7 to 7.3 min; $**P < 0.01$ from 7.7 to 12 min.

(28) (Fig. 4 *A* and *B*). In this system, transgenic zebrafish with GFP-positive motor neurons and axons are injected at the one- or two-cell stage with *smn* MO. At 28 h postfertilization, fish are classified as normal, mildly, moderately, or severely affected based on the motor axon defects (29). Such a classification of SMN-deficient motor neurons allows a rapid readout for modifier genes of SMN (27, 29). To test whether cpg15 might modify *Smn* deficiency in vivo, we overexpressed a human cpg15 construct in SMN-depleted zebrafish. *Smn* morphants were categorized as 19.7% ± 2.4% severe, 20% ± 0.6% moderate, 26% ± 1.6% mild, and 34% ± 1% unaffected (Fig. 4*D*). After injection of cpg15 mRNA together with *smn* MO, we observed a partial rescue of the motor axon phenotype, with only 11% ± 3% severe, 16% ± 1.6% moderate, 22.7% ± 7.5% mild, and 50% ± 7.9% unaffected (Fig. 4 *C* and *D*; $P < 0.01$). Partial rescue of the motor neuron axonal deficits observed in *Smn*-deficient zebrafish by *CPG15* supports the hypothesis that CPG15 is an important downstream effector of SMN and may serve as a modifier gene of SMA pathology in humans.

Discussion

Currently, MS-based proteomics is the most sensitive and comprehensive method for characterizing protein complexes. It is especially applicable to the study of low- abundance complexes, such as the SMN complex in neurons, where the starting material is limited. Our MS analysis of SMN interactions in neurons allowed us to isolate in vivo associations of the SMN protein in a quantitative manner. Our MS and co-IP data demonstrate a strong interaction between SMN and HuD in spinal motor neuron axons. They also show that cpg15 mRNA is a target of the SMN–HuD complex in neurons, such that a loss of SMN leads to a reduction in cpg15 mRNA level, and that the axonal defects observed in SMA zebrafish are rescued by the overexpression of human CPG15. Our findings elucidate an additional mechanism by which SMN deficiency may lead to abnormal axons.

The SMN protein interacts with several proteins in neurons, many of which have ubiquitous functions, such as pre-mRNA splicing, RNA metabolism and helicase activity, E2-dependent transcriptional activation, and mRNA transport (30). The best-characterized function of SMN is its role in pre-mRNA splicing, where it forms a stable and stoichiometric complex with Gemins 2–8 to regulate the assembly of snRNPs and their subsequent transport into nuclei for target-specific pre-mRNA splicing. snRNPs are the major components of the spliceosome that consists of Smith antigen (Sm), Sm-like (LSm) proteins, and U small nuclear RNAs (30). Interestingly, the axonal SMN complex is devoid of Sm proteins (2) and instead interacts with LSm proteins, suggesting a role in the assembly of RNP complexes important for mRNA transport (31). Furthermore, SMN can form a complex with hnRNP-Q/R protein, an RNP that appears to regulate axonal transport of β-actin mRNA (4, 9). In the present study, using MS and reciprocal co-IP analysis on neuronal tissues, we identified HuD as an RBP that strongly associates with SMN in motor neuron axons. Our findings provide further support for the hypothesis that SMN can associate with multiple RBPs to regulate axonal mRNA levels in neurons, and that the different SMN–RBP complexes may be defined by their mRNA contents.

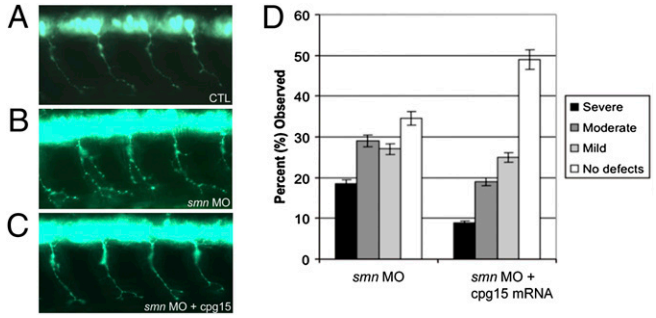

Fig. 4. Cpg15 partially rescues SMN deficiency. (*A–C*) Lateral-view representative images of *Tg(hb9:GFP)* embryos at 28 hpf uninjected (*A*), injected with 9 ng of *smn* MO (*B*), and injected with 9 ng of *smn* MO and 200 ng of full-length human cpg15 mRNA (*C*). (*D*) Full-length human cpg15 rescues motor axon defects caused by a reduction of Smn in zebrafish. *Tg(hb9:GFP)* zebrafish injected with 9 ng of *smn* MO and scored at 28 hpf using previously published criteria (29) resulted in a distribution of fish with motor nerve defects (n = 260 fish, 5,200 nerves, three injections). Coinjection of 9 ng of *smn* MO with 200 pg of full-length human *cpg15* mRNA was able to partially rescue the nerve defects (n = 326 fish, 6,520 nerves; $P < 0.01$). The distribution of larval classifications (severe, moderate, mild, and no defects) was analyzed using the Mann–Whitney nonparametric rank test.

Our analysis of known HuD-associated mRNAs in neurons identified cpg15 mRNA as a highly abundant mRNA in HuD IPs compared with other known targets of HuD, such as GAP43 and Tau. Given the role of SMN–hnRNP–β-actin mRNA interaction in targeting the β-actin mRNA to the axons, a similar mechanism appears to be at work with respect to the SMN/HuD protein complex and cpg15 mRNA. Using the Boyden chamber system, we found cpg15 mRNA expression in both the cell body and neurites along with β-actin and GAPDH mRNAs. CPG15 appears to be a crucial downstream effector of SMN in neurons and may play a role in SMA disease by regulating axon extension and axon terminal differentiation. Whether this role is cell-autonomous or not is not clear, however. CPG15 previously has been shown to function noncell-autonomously to regulate the growth and maturation of neighboring dendritic and axonal arbors (32, 33). Overexpression of CPG15 in SMA zebrafish motor neurons may exert nonautonomous effects by interacting with its receptor on neighboring cells supporting axonal growth and leading to partial rescue of the SMA phenotype. Identification of the CPG15 receptor would provide important information to distinguish between these possible mechanisms. In addition, further analyses are needed to determine whether motor neuron deficits and lethality in mouse SMA models can be rescued by CPG15 overexpression.

While this manuscript was being revised, two other groups reported an interaction between HuD and SMN, lending further support to our findings for a possible role of HuD in SMA pathology and the role of SMN–HuD interaction in mRNA transport/stability in neurons. Hubers et al. (34) provided substantial evidence that methylation of HuD by coactivator-associated arginine methyltransferase 1 regulates the ability of HuD to bind to target mRNAs and SMN. Thus, it would be interesting to explore whether cpg15 mRNA expression and axonal transportation are also affected by HuD methylation. Fallini et al. (35) demonstrated that SMN and HuD are transported together in motor neurons, and that SMN-deficient motor neurons have decreased amounts of HuD and poly(A) mRNA in their axons. Our results further highlight the role of SMN–HuD interaction and identify cpg15 mRNA as a candidate mRNA that modifies SMA pathology. Together, these findings support the notion that SMN forms other RNP complexes in motor neurons that may serve to regulate stability and transport of a subset of target mRNAs into axonal terminals. Characterization of the SMN–HuD complex will enable identification of the full repertoire of associated axonal mRNAs whose transport and/or stability are controlled by SMN and SMN-associated RBPs. Understanding such interactions and their motor neuron–specific dynamics will bring new insight into the mechanisms of SMN and its role in disease pathology.

Materials and Methods

See *SI Materials and Methods* for further experimental details.

Animals. All experimental procedures were performed in compliance with animal protocols approved by the Institutional Animal Care and Use Committees at Children's Hospital Boston and Ohio State University.

Immunofluorescence and Colocalization Analysis. E13 mouse motor neurons were isolated and grown in culture media for 5 d, as described in *SI Materials and Methods*. After 5 DIV, dissociated E13 mouse motor neurons were fixed, permeabilized in PBS Tween-20 (PBST), blocked in block buffer [5% normal goat serum (NGS) with 1× PBST], and incubated with primary antibodies diluted in block buffer at 4 °C overnight and then with secondary antibodies diluted in block buffer for 5 h. Coverslips were mounted with Prolong-Gold mounting media with DAPI (Invitrogen). The identity of motor neurons was verified by morphology and immunostaining with mouse choline acetyltransferase (ChAT; 28C4, ab78023; Abcam). At 5 DIV, >90% of the cells stained positive for anti-mouse ChAT (Fig. S1). Monoclonal mouse antibody to HuD (E-1, sc28299), monoclonal mouse synaptophysin antibody (mab5258; Millipore), rabbit antibody to SMN (H-195, sc15320), and goat antibody to HB9 were used to detect colocalization of endogenous proteins in motor neuron axons. The specificity of mouse HuD was confirmed by Western blot analysis; the polyclonal rabbit SMN antibody also was confirmed on lysates obtained from control and shSMN-infected neurons (Fig. S6). Secondary antibodies to rabbit Alexa Fluor 488 (Invitrogen), rabbit Alexa Fluor 594 (Invitrogen), and goat Cy5 (Abcam) were used to detect respective primary antibodies. Mouse antibody to Tau (mab3420; Millipore) was used to detect localization of SMN in the axons, and goat anti-Tau (C-17, sc1995) was used to detect localization of HuD in the axons (Fig. S2). Fluorescence imaging was visualized using a Zeiss LSM510 Meta NLO confocal microscope at a 63× magnification with zoom. Axons were imaged in either green (Alexa Fluor 488) or red (Alexa Fluor 594) channels along the *z*-axis (nine or ten 0.1-μm sections). Colocalization was measured using Zeiss LSM510 Imaging software, with the signal from individual puncta from each channel represented as peak intensity with a threshold value determined by the software. All of the green (SMN) puncta were counted, and all red puncta (representing either endogenous HuD or synaptophysin proteins) that fully overlapped with green puncta (representing endogenous SMN protein) were counted individually along the length of the axon (n = 10 for HuD vs. SMN colocalization and n = 7 for synaptophysin vs. SMN colocalization). The percentage of colocalization of SMN with HuD or synaptophysin was calculated, and the significance of the dataset was analyzed using the unpaired Student t test with Prism software.

Zebrafish Morpholino and Overexpression Experiments. Human *cpg15* was amplified with forward primer 5′-GATGGATCCCTATGGGACTTAAGTTG-3′ and reverse primer 5′-GATGAATTCTCAGAAGGAAAGCCAGG-3′ and then subcloned into the pCS2$^+$ vector using BamHI and EcoRI restriction sites. Plasmid DNA was linearized with NotI, and capped RNA was generated using the Sp6 mMESSAGE mMACHINE kit (Ambion) following the manufacturer's protocol. One- or two-cell stage *Tg(hb9:GFP)* (36) embryos were injected with ∼9 ng of *smn* MO (28) with or without 200 pg of synthetic human *cpg15* RNA using an MPPI-2 pressure injector (Applied Scientific Instrumentation). Three separate experiments were performed, with at least 50 embryos analyzed per experiment. At 28 h postfertilization (hpf), live embryos were anesthetized with tricaine and mounted on glass coverslips for observation with a Zeiss Axioplan2 microscope. Motor axons were scored as described previously (see table 1 in ref. 29). In brief, motor axons innervating the mid-trunk (myotomes 7–16) were examined under the compound microscope. Normal motor axon morphology is stereotyped along the trunk, and decreasing Smn levels caused motor axon truncations and abnormal branching. We defined abnormal branching as extra branches along the entire length of the motor axon or excessive branching at the distal region of the motor axon. We classified fish as having severe, moderate, mild, or no defects based on the character of the motor axon defects. Fish with at least 20% severe axon defects (i.e., truncations or truncation with abnormal branching) or 40% moderate defects (i.e., abnormal branching without truncations, "wishboned" axons) were classified as severe. Fish classified as moderate had 10% severe defects, 20–40% moderate defects, or >40% mild defects (i.e., axons lacking sterotyped

morphology but not abnormally branched). Fish classified as mild had 10% moderate defects and 20–40% mild defects. Each side of the fish was scored (20 total motor axons), and a classification was generated from the combined motor axon defects. Data were analyzed and statistical significance was determined using the Mann–Whitney nonparametric rank test.

ACKNOWLEDGMENTS. We thank Thomas Schwarz, Elly Nedivi, and members of the M.S. laboratory for critical reading of the manuscript, and Xianhua Chen for sharing unpublished data. We also thank John Sauld, Zachary Waldon, Saima Ahmed, Abbey Sadowski, and Samuel Goldman for technical assistance. This work was supported in part by National Institutes of Health Grants R01 NS66973 (to J.A.J.S. and M.S.), NS041596 (to J.L.T.), and NS050414 (to C.E.B.); the SMA Foundation grants (to C.E.B. and M.S.), American Academy of Neurology Foundation, the Slaney Family Fund and Children's Hospital Boston Translational Research Program grants (to M.S.), and Children's Hospital Boston Mental Retardation and Developmental Disabilities Research Center Grant P30 HD18655). B.A. is supported by a grant from the William Randolph Hearst Foundation. M.J.K. is supported by a grant from the Harvard NeuroDiscovery Center.

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