

Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons

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Spinal muscular atrophy (SMA) is a neurodegenerative disease primarily affecting spinal motor neurons. It is caused by reduced levels of the survival of motor neuron (SMN) protein, which plays an essential role in the biogenesis of spliceosomal small nuclear ribonucleoproteins in all tissues. The etiology of the specific defects in the motor circuitry in SMA is still unclear, but SMN has also been implicated in mediating the axonal localization of mRNA-protein complexes, which may contribute to the axonal degeneration observed in SMA. Here, we report that SMN deficiency severely disrupts local protein synthesis within neuronal growth cones. We also identify the cytoskeleton-associated growth-associated protein 43 (GAP43) mRNA as a new target of SMN and show that motor neurons from SMA mouse models have reduced levels of GAP43 mRNA and protein in axons and growth cones. Importantly, overexpression of two mRNA-binding proteins, HuD and IMP1, restores GAP43 mRNA and protein levels in growth cones and rescues axon outgrowth defects in SMA neurons. These findings demonstrate that SMN plays an important role in the localization and local translation of mRNAs with important axonal functions and suggest that disruption of this function may contribute to the axonal defects observed in SMA.

Key words: GAP43; local translation; motor neuron; RNA trafficking; SMA; SMN

Significance Statement

The motor neuron disease spinal muscular atrophy (SMA) is caused by reduced levels of the survival of motor neuron (SMN) protein, which plays a key role in assembling RNA/protein complexes that are essential for mRNA splicing. It remains unclear whether defects in this well characterized housekeeping function cause the specific degeneration of spinal motor neurons observed in SMA. Here, we describe an additional role of SMN in regulating the axonal localization and local translation of the mRNA encoding growth-associated protein 43 (GAP43). This study supports a model whereby SMN deficiency impedes transport and local translation of mRNAs important for neurite outgrowth and stabilization, thus contributing to axon degeneration, muscle denervation, and motor neuron cell death in SMA.

Introduction

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by progressive denervation of skeletal muscles, which results in muscle weakness, paralysis, and death due to

respiratory failure (Crawford and Pardo, 1996). The primary pathology in SMA is a developmental neuromuscular junction (NMJ) synaptopathy (Kariya et al., 2008; Dupuis and Echaniz-Laguna, 2010) followed by degeneration of motor neurons. Axonal degeneration precedes motor neuron death (Monani et al., 2000; Cifuentes-Diaz et al., 2002) and defects at the NMJ are the earliest events detected in SMA mouse models (Murray et al., 2008; Ling et al., 2010; Ling et al., 2012; Martinez et al., 2012; Goulet et al., 2013; Ruiz and Tabares, 2014).

The molecular defect causing SMA is a reduction in the levels of the survival of motor neuron (SMN) protein due to mutations in the *SMN1* gene locus (Lefebvre et al., 1995). However, the downstream molecular pathways leading to motor neuron degeneration are still unknown. SMN is a ubiquitously expressed protein with a well characterized essential function in the assembly of small nuclear ribonucleoproteins (snRNPs), the key components of spliceosomes (Gubitz et al., 2004; Battle et al., 2006;

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Coady and Lorson, 2011; Workman et al., 2012). Recent studies have shown that, beyond its role in snRNP assembly, SMN is essential for the biogenesis of U7 snRNPs and 3'-end formation of histone mRNAs (Tisdale et al., 2013) and may have a more general role as a molecular chaperone for the assembly of various RNP complexes (Friesen and Dreyfuss, 2000; Brahms et al., 2001; Azzouz et al., 2005; Pellizzoni, 2007; Boulisfane et al., 2011; Lotti et al., 2012; Li et al., 2014). In particular, SMN has been suggested to play a role in regulating the localization of mRNAs and mRNA-binding proteins (mRBPs) in axons (Akten et al., 2011; Fallini et al., 2011; Hubers et al., 2011; Sanchez et al., 2013; Fallini et al., 2014). The mislocalization of β -actin mRNA in SMA motor neurons was the first example (Rossoll et al., 2003); however, tissue-specific deletion of the β -actin locus in motor neurons did not affect their survival nor the formation and maintenance of the NMJ (Cheever et al., 2011). This observation suggests that deficiency of multiple mRNAs may underlie SMA pathogenesis, which is consistent with our previous report of deficient poly(A) mRNA localization in the axon of SMN-depleted motor neurons (Fallini et al., 2011) and recent RNAseq analysis of axonal mRNAs in SMA motor neurons (Saal et al., 2014). Defects in mRNA localization are accompanied by a similar decrease in axonal levels of the SMN-interacting mRBPs HuD and IMP1 (Fallini et al., 2011; Fallini et al., 2014). IMP1, also known as zipcode binding protein 1 (ZBP1), is a KH domain RNA-binding protein required for the localization and local translation of several mRNAs, including β -actin and growth-associated protein 43 (GAP43) mRNA (Donnelly et al., 2013). IMP1 associates in actively transported RNA granules with the ELAV-like RNA-binding protein HuD and cooperatively regulates the stability and axonal localization of *Tau* and *GAP43* mRNA (Atlas et al., 2007; Yoo et al., 2013). HuD and *GAP43* mRNA colocalize in RNA granules within axons and growth cones (Smith et al., 2004). SMN interaction with HuD was previously shown to play a role in *neuritin/cpg15* mRNA localization, which is altered in SMA (Akten et al., 2011).

In this study, we first show that the reduction of poly(A) mRNA levels in SMA axons directly results in deficiency of axonal protein synthesis. We identify *GAP43* as an mRNA that is reduced and mislocalized in SMA motor neurons and test the hypothesis that impairments in axonal localization of *GAP43* mRNA and protein plays a role in SMA. Our results show that reduced *GAP43* mRNA and protein levels in SMA motor neuron growth cones can be restored by increasing the expression of two regulatory mRBPs, HuD and IMP1, which is sufficient to rescue the impairment in axon outgrowth observed in SMA motor neurons. Together, these findings support a model of dysregulated local translational as a contributing mechanism to SMA pathogenesis.

Materials and Methods

Motor neuron culture and transfection. Primary motor neurons from wild-type (WT) and SMA (*Smn*^{-/-}; *hSMN2*; stock number #005024; Jackson Laboratories) embryonic day 13.5 (E13.5) mouse embryos of either sex were isolated, cultured, and transfected by magnetofection as described previously (Fallini et al., 2010; Fallini et al., 2011). Cells were fixed at 3–5 d *in vitro* (DIV) or 2–3 d after transfection as indicated, and processed for quantitative FISH (Q-FISH) or immunostaining. Monomeric green (GFP) or red (mCherry) fluorescent proteins were fused to murine SMN (Fallini et al., 2010), human HuD (Fallini et al., 2011), and rat IMP1 (Fallini et al., 2014) cDNAs. A flexible linker [(SGGG)₃] was inserted between all the fusion partners to facilitate correct protein folding. The pGIPZ shRNA vectors targeting SMN sequence (shSMN) and a nonsilencing control (shCtrl, RHS4346) were obtained from Open Biosystems (Fallini et al., 2011). For axon length analysis, cells were trans-

ected with GFP alone to label the whole axon and to identify individual cells.

FISH and immunofluorescence. Motor neurons were fixed for 15 min with 4% paraformaldehyde in PBS. FISH was performed as described previously (Fallini et al., 2011) with some modifications. Briefly, fixed motor neurons were rinsed in PBS containing 5 mM MgCl₂ and equilibrated in 1× SSC buffer for 10 min. Cells were then washed in 10% formamide (Sigma-Aldrich) for 10 min before preincubation in hybridization buffer (20% dextran sulfate, 4× SSC, 4 mg/ml BSA, 20 mM ribonucleoside vanadyl complex, and 10 mM sodium phosphate buffer, pH 7.0) at 37°C for 1.5 h. Probes (1 μ l) were resuspended with 10 μ g each of *E. coli* tRNA and salmon sperm DNA in 50 μ l of hybridization buffer and incubated with the coverslips at 37°C overnight. Stellaris FISH probes for *GAP43* and β -actin directly labeled with Quasar570 and Quasar670, respectively, were obtained from Biosearch Technologies. A Cy3-labeled oligo dT probe (Biosearch Technologies) was used to detect poly(A)-positive mRNAs. The specificity of the probes was demonstrated using a GFP control probe (see Fig. 4C). For immunofluorescence assays, fixed motor neurons were incubated overnight at 4°C with *GAP43* (Epitomics, 1:250) and SMN (BD Biosciences, 1:500) antibodies in blocking buffer (5% BSA, 1×PBS). Cy3-, Cy2-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were incubated for 1 h at room temperature.

Image acquisition and analysis. For high-resolution imaging, a 60× objective (1.4 numerical aperture) was used. Z-series (5–10 sections, 0.2 μ m thickness) were acquired with an epifluorescence microscope (Ti; Nikon) equipped with a cooled CCD camera (HQ2; Photometrics). For low-magnification imaging, a 10× or 20× phase objective was used and single optical slices were acquired. Z-stacks were deconvolved (Autoquant X2; Media Cybernetics) and analyzed using Imaris software (Bitplane). For the analysis of fluorescence intensity, a 70–80 μ m segment of the axon starting 20 μ m from the cell body was analyzed. Background fluorescence was subtracted in all channels and an additional threshold was applied to discriminate between signal and noise. Axon length measurements were performed as described previously (Fallini et al., 2012b).

Protein extraction and Western blot. Brain and spinal cord tissue isolated from E12 mouse embryos of either sex was homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2% Triton X-100, protease inhibitors) and sonicated on ice 3 × 10 min. Proteins were separated on a 10% polyacrylamide-SDS gel and hybridized with primary antibodies directed against SMN (BD Bioscience, 1:500), *GAP43* (Epitomics, 1:500), actin (Sigma-Aldrich, 1:1000), and tubulin (Sigma-Aldrich, 1:1000). IRDye-conjugated secondary antibodies (LI-COR) were used for detection. The intensity of the protein bands was quantified using ImageJ.

Metabolic labeling of newly synthesized proteins with AHA. Primary E18 cortical neurons were transfected via nucleofection (Lonza) with either shCtrl or shSMN plasmids and plated in PDMS microfluidic chambers (Xona) mounted on poly-D lysine-coated cover glass. Cells were grown until axons crossed into the axonal chamber (DIV4), and methionine-free DMEM (Invitrogen) supplemented with B27 was then added to both the axonal and cell body compartments for 1 h. After methionine starvation, 2 mM L-azidohomoalanine (AHA) was added to only the cell body or axonal compartment to restrict AHA labeling locally and spatially to individual compartments with or without 40 μ M anisomycin to inhibit protein synthesis. Media volume was higher in compartments without AHA added to prevent diffusion of AHA. Cells were washed and fixed after 2 h and AHA incorporation was detected with Alexa Fluor 647-conjugated alkyne using Click-iT chemistry (Invitrogen) according to the manufacturer's recommendations.

Statistical analysis. Experimental data were analyzed for statistical significance using the Prism 6 (GraphPad) software. Individual values were normalized to the mean of the control sample (e.g., WT cells) and measurements from at least three individual experiments were pooled together. For normally distributed data, Student's *t* test or one-way ANOVA with Dunnett's *post hoc* test were used. For axon length analysis, axon measurements from each individual experiment were normalized to the mean of the control sample (i.e., WT cells) and values from four separate experiments were pooled together. The distribution of the data across the whole population was analyzed using cumulative frequency plots that display the frequency of occurrence (*y*-axis) of axonal length values (*x*-axis) that were equal to or less

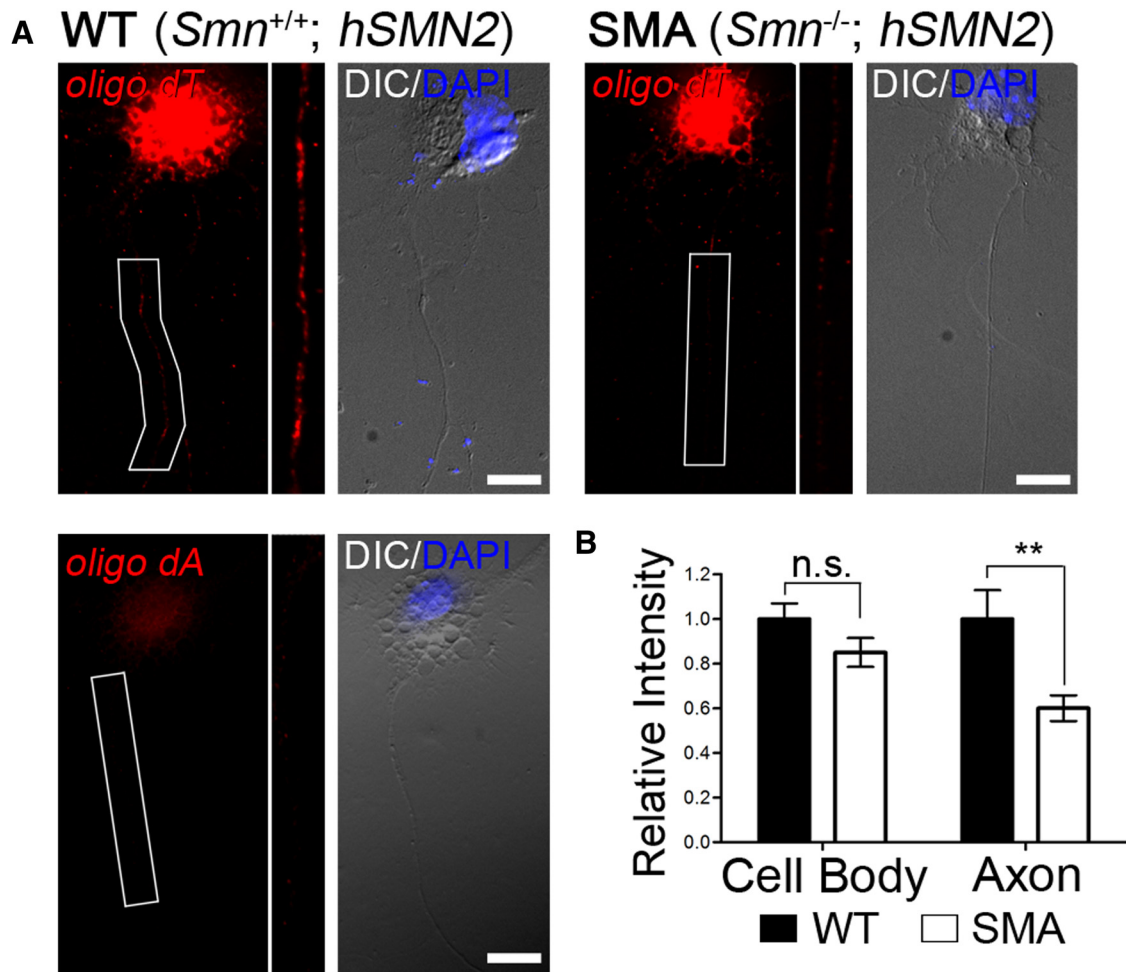


Figure 1. SMA motor neurons have reduced levels of axonal poly(A) mRNAs. **A**, Motor neurons isolated from SMA and WT embryos were hybridized with an oligo dT probe to detect poly(A) mRNAs. An oligo dA probe was used as a negative control. Scale bar, 10 μ m. **B**, Fluorescence intensity of the FISH signal in the cell body and axon was quantified and compared between WT and SMA cells. A significant reduction was observed in the axon, whereas no difference was detected in the cell body. Error bars indicate mean \pm SEM (Student's *t* test; *n* = 40 cells from three independent experiments; ***p* < 0.01; ns, not significant).

than a reference value. The Kolmogorov–Smirnov test was used to determine whether the distributions from the different conditions were significantly different from the control population (i.e., WT). For all analyses, significance was defined as *p* < 0.05.

Results

SMN deficiency affects mRNA distribution and translation at the growth cone

Deficiency of SMN protein in shRNA-treated motor neurons (Fallini et al., 2011) and motor neurons derived from an SMA mouse model have significantly reduced poly(A) mRNA content in the axons, but not in the cell bodies (Fig. 1). This observation led us to hypothesize that reduced axonal transcript levels may cause a compartmentalized reduction in local protein translation. To test this hypothesis, cortical neurons that readily grow and project axons across compartmentalized microfluidic chambers were transfected with shRNA vectors to knock down SMN protein expression (shSMN; Fig. 2*A, B*; Fallini et al., 2011). Newly synthesized proteins in the cell body or growth cone compartment were detected using FUNCAT (fluorescent noncanonical amino acid tagging; Tom Dieck et al., 2012). The methionine analog AHA was added to either the cell body or the axon side for metabolic labeling. Click chemistry was used to fluorescently tag the incorporated AHA and newly synthesized proteins were

quantified using high-resolution fluorescence microscopy. Whereas no effect was observed on overall protein synthesis in the cell soma, a 60% reduction in the levels of AHA-labeled proteins was detected in neuronal growth cones (Fig. 2*C–E*). Although we cannot exclude that subtle changes in the translational levels in the cell soma were masked due to the abundance of newly synthesized proteins, these data suggest no global change in protein synthesis, but rather more spatially restricted local changes distally possibly due to defective mRNA localization. Together, these results demonstrate that SMN deficiency leads to defective mRNA localization and consequent impairment in local protein synthesis in the distal axon, thus possibly contributing to the axonal phenotype in SMA.

GAP43 mRNA is mislocalized in SMA motor neurons

The axon outgrowth defect in SMA motor neurons suggests the involvement of other transcripts in addition to β -actin because axonally synthesized β -actin is mostly associated with axonal branching rather than elongation (Donnelly et al., 2013). Therefore, we investigated whether axon-growth-promoting mRNAs are mislocalized in SMA motor neurons. In particular, we focused on GAP43 mRNA, a known target of the mRBPs HuD and IMP1. Two cellular models were used to assess the effects of SMN

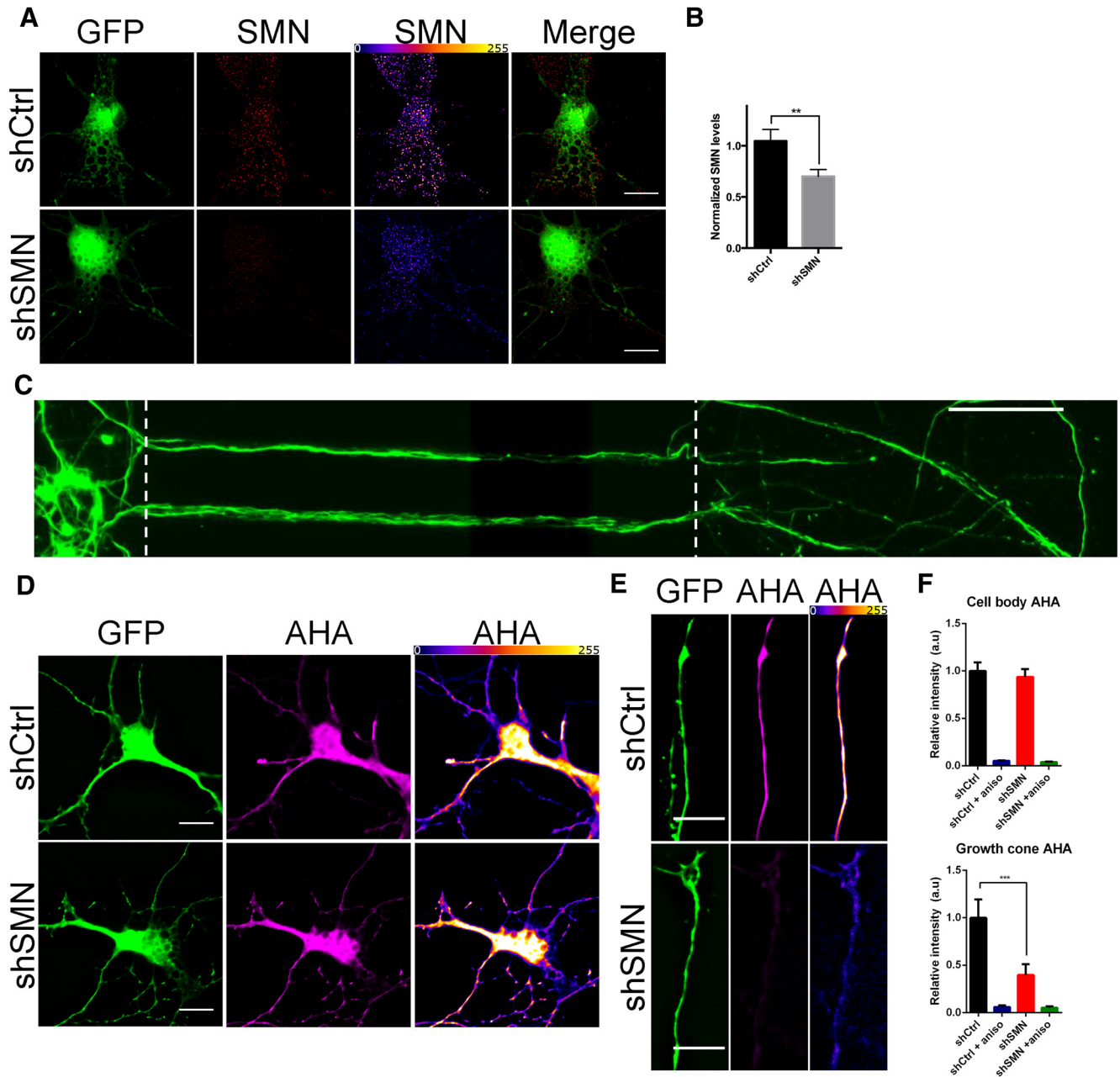


Figure 2. SMN deficiency causes reduced local protein synthesis in axonal growth cones. **A, B**, SMN knock-down efficiency (red) was quantified in cortical neurons transfected with an shRNA directed against SMN (shSMN) versus control cells (shCtrl). GFP (green) was used to identify transfected cells. Error bars indicate mean \pm SEM (Student's *t* test, $n = >30$ cells/condition from three independent experiments, $**p < 0.001$). Cells in **A, D**, and **E** are also displayed as pseudocolored heat map of pixel intensity, with warm colors denoting higher signal (0–255). Scale bar, 10 μ m. **C**, Cortical neurons grown in microfluidic chambers stained for tubulin. Scale bar: 100 μ m. **D, E**, The methionine analog AHA was added to either the cell body or the axon side for metabolic labeling. SMN depletion leads to decreased incorporation of AHA in growth cones (**E**) of cortical neurons with no change in the cell bodies (**D**). Scale bar, 10 μ m. **F**, Quantification of AHA staining in the cell body and growth cone from shCtrl and shSMN neurons in the presence or absence of anisomycin to block protein synthesis. Error bars indicate mean \pm SEM (one-way ANOVA with Dunnett's post hoc test, $n = >30$ cells/condition from three independent experiments, $***p < 0.001$).

deficiency on *GAP43* mRNA and protein localization: (1) WT motor neurons in which SMN levels were acutely reduced by shRNA and (2) motor neurons isolated from SMA mouse embryos (E13.5; *Smn*^{-/-}; *hSMN2*) that have chronically low amounts of full-length SMN protein. The localization and abundance of *GAP43* and β -actin mRNAs were assessed by Q-FISH. A striking reduction in the levels of both mRNAs was observed in the axons and growth cones of shSMN-transfected motor neurons 5 d after transfection (Fig. 3). No difference was detected in the cell bodies. Similarly, after 3 DIV, motor neurons obtained from SMA mouse embryos showed a 26% and 28% reduction,

respectively, in the levels of *GAP43* and β -actin mRNAs in the proximal axonal segment compared with WT (*Smn*^{+/+}; *hSMN2*) controls (Fig. 4). A clear trend toward reduction was detected in SMA growth cones (*GAP43*: 0.70 ± 0.17 ; β -actin: 0.64 ± 0.07), although it did not reach statistical significance, and no difference was observed in the cell body. However, when motor neurons were analyzed at 5 DIV, the levels of *GAP43* and β -actin mRNAs were significantly reduced also in the cell body by 26% and 25%, respectively (Fig. 4E). These results suggest that the general reduction of *GAP43* and β -actin mRNA levels in cell bodies is subsequent to the early axonal localization defect.

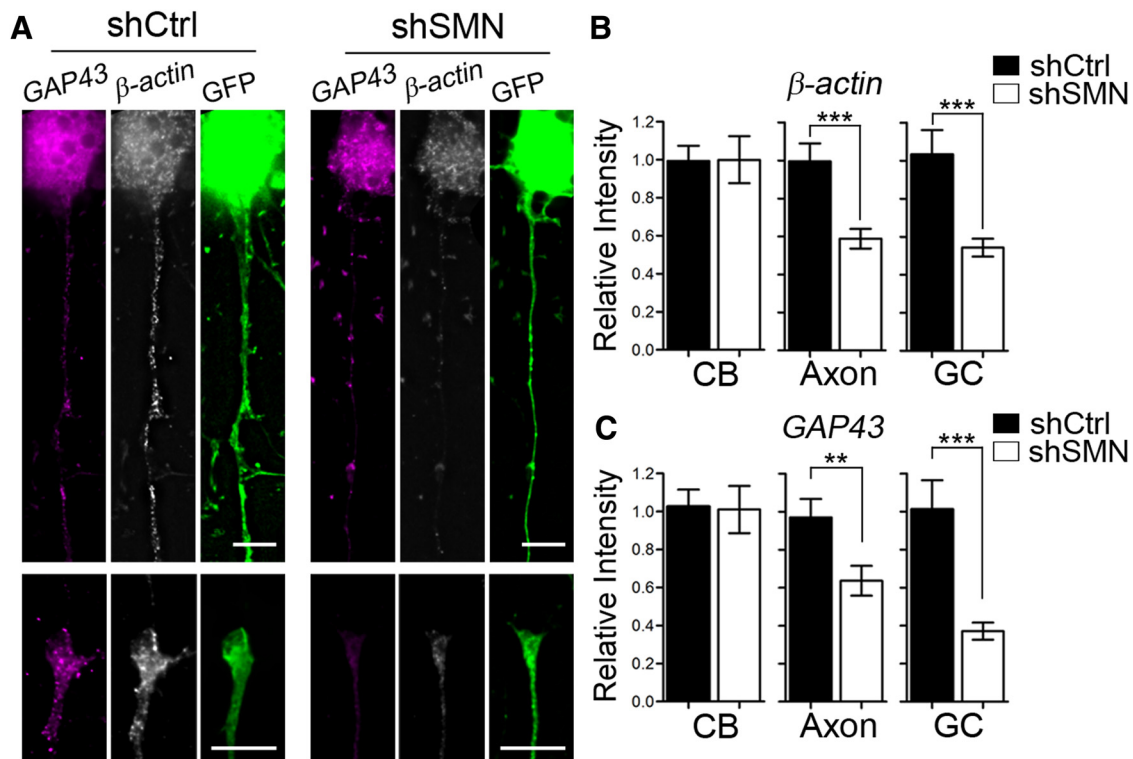


Figure 3. *GAP43* and β -actin mRNAs are reduced in axons and growth cones of SMN deficient motor neurons. **A**, Primary motor neurons were transfected with an shRNA construct directed against SMN (shSMN) or a control vector (shCtrl). Five days after transfection, cells were fixed and hybridized with probes specific for *GAP43* (magenta) and β -actin (white) mRNA. GFP (green) identifies transfected cells. Scale bar, 10 μ m. **B**, **C**, Fluorescence intensity was quantified in the cell body, axon, and growth cone and compared between shSMN and shCtrl cells. A significant reduction in both *GAP43* (**C**) and β -actin (**B**) mRNA levels was observed in the axon and growth cone. Error bars indicate mean \pm SEM (Student's *t* test; $n = 30$ cells from three independent experiments; *** $p < 0.01$, *** $p < 0.001$). Scale bar, 10 μ m.

GAP43 protein is reduced in SMA growth cones

To investigate whether a reduction in *GAP43* mRNA levels in the axon of SMA motor neurons is associated with a similar decrease in the levels of GAP43 protein, we performed quantitative immunofluorescence in primary motor neurons isolated from SMA embryos. We found that *GAP43* mRNA decreased localization is sufficient to reduce its protein levels at the growth cone (Fig. 5*A,B*). SMA motor neurons at 3 DIV, when no reduction of *GAP43* mRNA levels is observed in the cell body, showed significantly lower levels of GAP43 protein at the growth cone compared with WT cells (0.69 ± 0.07 SMA vs 1.0 ± 0.12 WT), whereas a nonsignificant 39% increase in the cell body was detected. At 5 DIV, GAP43 protein levels were reduced also in the cell body (data not shown). Similar results were observed in shSMN-transfected motor neurons (Fig. 5*C,D*). However, no difference in GAP43 protein levels was detected in whole-brain and spinal cord lysates by Western blotting (Fig. 5*E*). These data suggest that SMN is important for the axonal localization of *GAP43* mRNA and protein, which, in the absence of SMN, are retained in the cell body and eventually degraded. A similar mild reduction in the overall mRNA levels associated with a more dramatic decrease in the axonal localization was observed for the SMN-associated mRNAs *Anxa2a* and *Cox4i2* (Rage et al., 2013).

Overexpression of IMP1 and HuD rescues GAP43 axonal deficiency

Because *GAP43* mRNA stability, transport, and translation are controlled by the mRBPs HuD and IMP1 (Yoo et al., 2013), the expression of which is reduced in SMA motor neurons, we hypothesized that restoring the levels of these mRBPs could rescue

the reduction of GAP43 levels at the axon tip. To test this possibility, we expressed GFP-tagged HuD and IMP1 in SMA motor neurons and quantified GAP43 protein levels 2 d after transfection (Fig. 6*A*). Under these conditions, we observed a significant increase in GAP43 protein at the growth cone to levels similar to WT cells (0.73 ± 0.09 GFP-HuD, 0.97 ± 0.18 GFP-IMP1 vs 1 ± 0.13 WT). Importantly, the rescue of GAP43 protein levels due to HuD and IMP1 expression was associated with a parallel increase in the levels of *GAP43* mRNA (0.92 ± 0.16 GFP-HuD, 1.19 ± 0.16 GFP-IMP1 vs 1.00 ± 0.09 WT; Fig. 6*B*), suggesting that overexpression of these mRBPs is sufficient to enhance *GAP43* mRNA transport and local translation. Because GAP43 acts as a positive regulator of axon outgrowth and growth cone stability, we investigated whether the rescue of GAP43 protein levels by IMP1 and HuD expression could also restore axon growth in SMA motor neurons. SMA motor neurons were transfected with fluorescently tagged mRBPs and the length of the main axon branch was measured 2 d after transfection. Although SMA motor neurons had significantly shorter axons compared with WT cells, the expression of mCherry-tagged HuD or IMP1 was able to fully rescue the axonal defect (Fig. 6*C*). Together, these results suggest that the reduction in the mRNA and protein levels of *GAP43*, and possibly other HuD/IMP1 targets in the axons and growth cones of SMA motor neurons, contribute to the axonal defects that characterize SMA pathology.

Discussion

The role of SMN as a spliceosome assembly factor has been characterized extensively (Gubituz et al., 2004; Battle et al., 2006; Coady and Lorson, 2011; Workman et al., 2012). However, the evidence

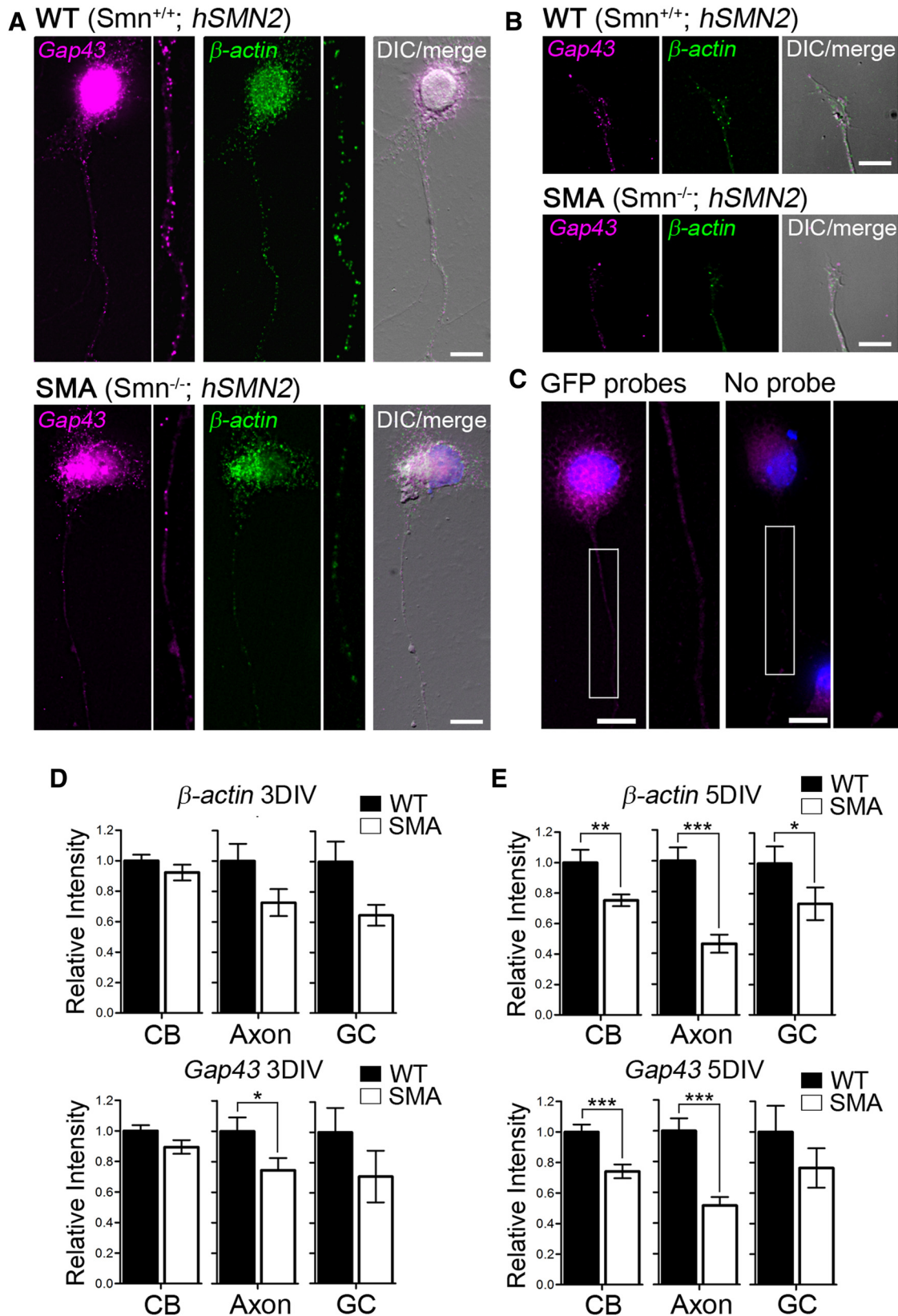


Figure 4. *GAP43* mRNA levels are reduced in axons, growth cones, and cell bodies during the development of SMA motor neurons. **A, B**, Q-FISH of *GAP43* (magenta) and β -actin (green) mRNA in WT and SMA motor neurons. Scale bar, 10 μ m. **C**, No probe and GFP probes were used as FISH controls. **D, E**, Quantification of fluorescence intensity in 3 and 5 DIV WT and SMA neurons in the cell body (CB), axon, and growth cone (GC). Error bars indicate mean \pm SEM (Student's *t* test; $n = 45$ – 65 cells from three independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

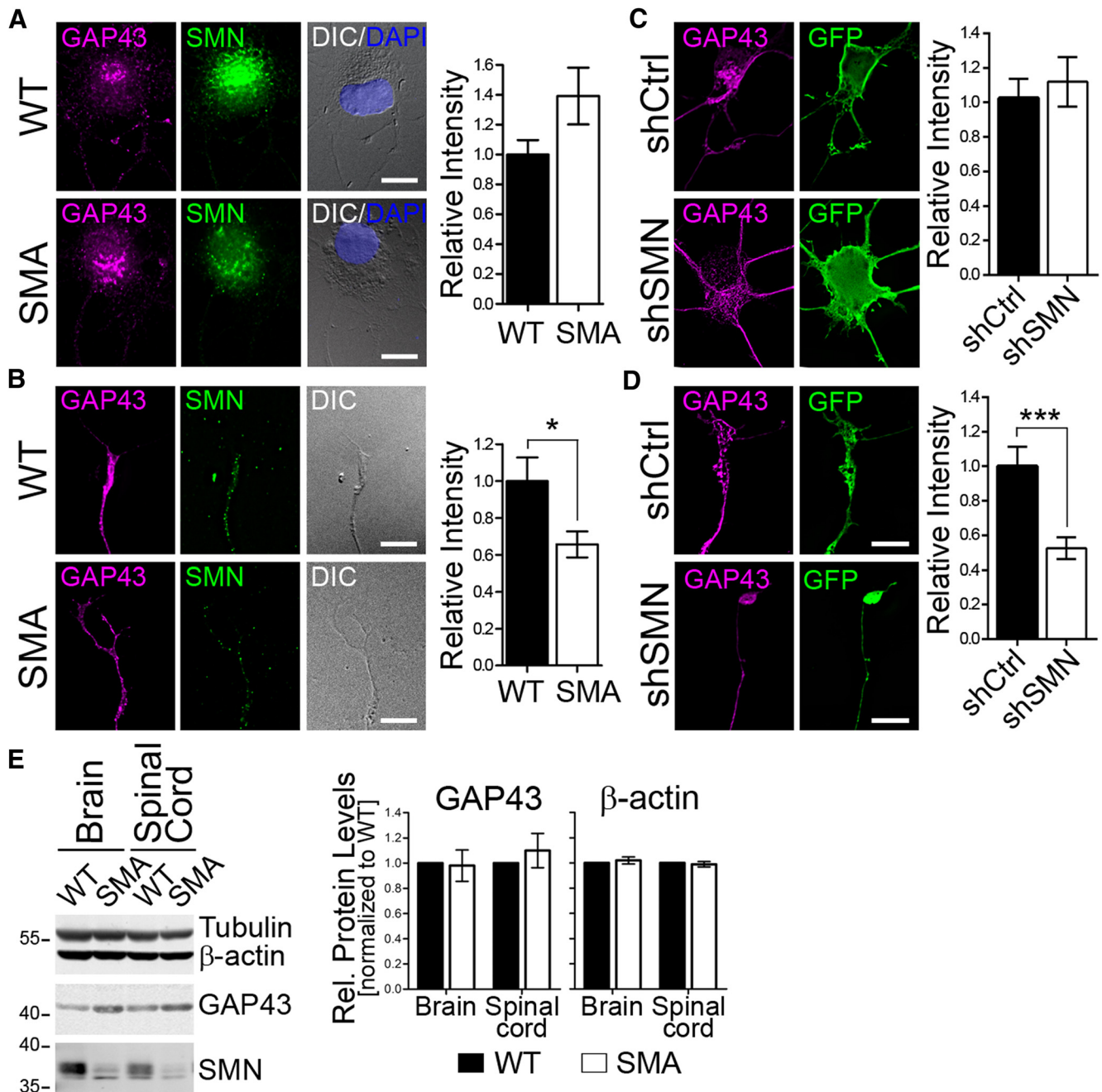


Figure 5. GAP43 protein levels are reduced in axonal growth cones of SMA motor neurons. **A, B**, Immunostaining and quantification of GAP43 levels (magenta) in the cell body (**A**) and growth cone (**B**) of SMA and WT motor neurons. Error bars indicate mean \pm SEM (Student's *t* test; $n = 43$; $*p < 0.05$). Scale bar, 10 μ m. **C, D**, Immunostaining and quantification of GAP43 protein levels (magenta) in the cell body (**C**) and growth cone (**D**) of motor neurons transfected with shRNA constructs. GFP expression (green) was used to identify transfected cells. Error bars indicate mean \pm SEM (Student's *t* test; $n = 40$ cells from three independent experiments; $***p < 0.001$). Scale bar, 10 μ m. **E**, Western blot analysis of GAP43 and β -actin protein levels in SMA tissue. SMN and Tubulin were used as controls. Error bars indicate mean \pm SEM ($n = 4$).

linking SMA pathogenesis to defects in mRNA splicing is still inconclusive. Over the past decade, we and others have demonstrated an additional function for SMN in the regulation of the axonal localization of ribonucleoproteins (mRNPs; Rossoll et al., 2002; Rossoll et al., 2003; Akten et al., 2011; Fallini et al., 2011; Hubers et al., 2011; Fallini et al., 2014). Two mRNPs, HuD (Akten et al., 2011; Fallini et al., 2011; Hubers et al., 2011) and IMP1 (Fallini et al., 2014), have been shown to interact with SMN and to depend on SMN for their axonal localization. Both HuD and IMP1 bind and control the localization, stability, and transport of, among others, GAP43, β -actin, tau, and neurtin/cpg15 mRNAs

(Atlas et al., 2007; Akten et al., 2011; Yoo et al., 2013). SMN deficiency leads to the dysregulation of not one, but several axonal mRNAs, including β -actin and neurtin/cpg15 (Rossoll and Bassell, 2009; Fallini et al., 2012a; Li et al., 2014). Recent work has further revealed impairments in the local translation of mTOR mRNA in SMA (Kye et al., 2014), suggesting general impairments in local protein synthesis. Here, we analyzed newly synthesized proteins in the cell body and growth cone to show directly that axonal, but not somatic, protein synthesis is reduced in SMA. We further show that in SMA motor neurons the localization of GAP43 mRNA and protein at the growth cone is impaired.

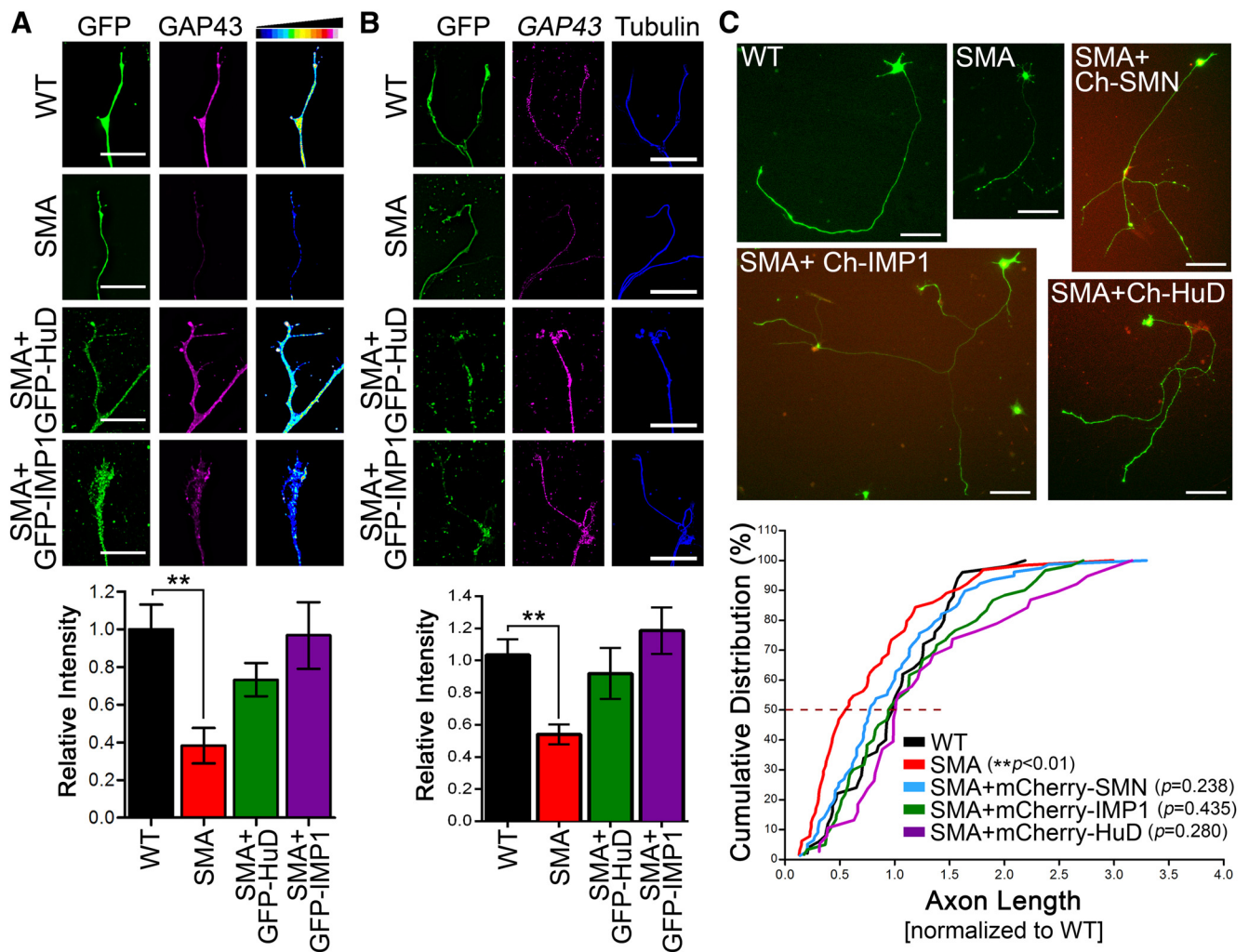


Figure 6. Overexpression of HuD and IMP1 restores GAP43 protein and transcript levels in growth cones, as well as axon outgrowth of SMA motor neurons. **A**, Immunostaining and quantification of GAP43 protein levels (magenta) in WT and SMA motor neurons with or without the expression of GFP-tagged HuD and IMP1 (green). Error bars indicate mean \pm SEM (one-way ANOVA and Dunnett's *post hoc* test; $n = 30$ growth cones from three independent experiments; $**p < 0.01$). Scale bars, $10 \mu\text{m}$. **B**, FISH and quantification of GAP43 mRNA levels (magenta) in WT and SMA motor neurons with or without the expression of GFP-tagged HuD and IMP1 (green). Tubulin (blue) was used to label the axons and growth cones. Error bars indicate mean \pm SEM (one-way ANOVA and Dunnett's *post hoc* test; $n = 46$ – 48 growth cones from three independent experiments; $**p < 0.01$). Scale bars, $10 \mu\text{m}$. **C**, Representative images and cumulative distribution analysis of axonal length of WT and SMA motor neurons with or without the expression of mCherry-tagged SMN, HuD, and IMP1. The y -axis represents the frequency of the occurrence of axonal length values equal or less than a reference value. For statistical analysis, the Komogorov–Smirnov test was used ($n = 38$ – 70 cells from three independent experiments). Scale bars, $50 \mu\text{m}$.

GAP43 is of special interest because this growth cone protein plays a key role in regulating growth cone stability and axon growth. GAP43, by stabilizing actin filaments (He et al., 1997), positively regulates neurite growth and pathfinding (Aigner et al., 1995; Strittmatter et al., 1995; Shen et al., 2002; Shen and Meiri, 2013), axon sprouting after injury (Donnelly et al., 2013; Yoo et al., 2013), and the maintenance of synaptic stability (Allegra Mascaro et al., 2013). Motor neurons derived from SMA animal models show impaired axon growth (McWhorter et al., 2003; Rossoll et al., 2003; Winkler et al., 2005; Ymlahi-Ouazzani et al., 2010) and inefficient sprouting (Cifuentes-Diaz et al., 2002; Murray et al., 2008) in response to muscle denervation. Together, these observations point to GAP43 as an intriguing novel target that may underlie axonal growth defects in SMA disease motor neurons. Furthermore, *in vivo* gene silencing of GAP43 in adult rodent climbing fibers demonstrates that this protein is essential not only during regeneration, but also for maintenance of axons under normal conditions (Grasselli et al., 2011). It is interesting that the mRNAs that have been shown to be misregulated in

SMN-deficient cells, such as GAP43 in the present study and the previously identified β -actin, tau, and neurtin/cpg15, code for proteins that are involved in the regulation of axonal growth and neuron plasticity during development and regeneration (Denny, 2006; Dent et al., 2011; Zhou and Zhou, 2014), processes that are altered in SMA.

We also show here that the overexpression of two regulatory mRBPs, HuD and IMP1, is sufficient not only to restore GAP43 levels at the growth cone, but also to rescue the axon outgrowth defects observed in SMA motor neurons. Importantly, our data show that local protein synthesis at the distal axon is reduced in SMA neurons, suggesting that GAP43 protein deficiency is due to its reduced mRNA transport and that mRBP expression restores GAP43 mRNA localization and local translation. This is further supported by the fact that the local synthesis at the growth cone of the GAP43 mRNA is required to enhance axon growth (Donnelly et al., 2013). Indeed, expression of a chimeric GAP43 mRNA in which the 3'UTR was replaced with a nonlocalizing sequence (i.e., γ -actin 3'UTR) was shown to have a minimal effect on axon

growth (Donnelly et al., 2013). These results provide further support for the hypothesis that SMA is at least partially caused by a disruption in the SMN-dependent regulation of axonal mRNA localization and local translation.

The reduction in the levels of functional SMN protein in motor neurons is associated with the axon-specific downregulation of several transcripts. Recent microarray analyses of axonal mRNAs performed in SMA motor neurons and NSC-34 cells demonstrated that molecular pathways such as synapse function and formation, protein translation, and mRNA binding were particularly affected (Saal et al., 2014). Interestingly, a large percentage of SMN-associated mRNAs are enriched in the axons, supporting SMN's role in axonal mRNA localization (Rage et al., 2013). Furthermore, these and similar studies suggest that the disruption of a single specific transcript may not lead to an SMA-like phenotype and, similarly, that restoring individual mRNAs may not be sufficient to fully rescue the SMA phenotype (Zhang et al., 2008; Fallini et al., 2011; Saal et al., 2014). Indeed, restoring *neurtin/cpg15* mRNA levels in zebrafish SMA motor neurons only partially rescued motor axon defects (Akten et al., 2011) and the motor-neuron-specific knock-out of the β -*actin* gene had no obvious phenotypic consequences in mice (Cheever et al., 2011). Our approach to attempting a phenotypic rescue via the expression of the regulatory mRBPs in SMA motor neurons was not only able to restore *GAP43* mRNA and protein levels, but also fully rescued the defects in motor axon outgrowth observed in SMA motor neurons. This is possibly due to the fact that HuD and IMP1 regulate the mRNA localization and translation of multiple transcripts encoding SMA-relevant modulators of cytoskeletal dynamics and function. Future work will be required to identify additional transcripts and their relative contribution to the rescue of the axonal phenotype.

Although we cannot exclude that HuD and IMP1 overexpression rescued the observed phenotypes by modulating proposed SMA-specific splicing defects or by affecting *SMN2* pre-mRNA splicing, this is unlikely. Both HuD and IMP1 mRBPs have very limited or no known effect on mRNA splicing and the large majority of HuD and IMP1 binding sites are located in the 3' and 5'-UTRs, rather than intronic sequences (Jønson et al., 2007; Bolognani et al., 2010; Bronicki and Jasmin, 2013). Furthermore, *SMN* mRNA was not detected as a target of either HuD or IMP1 in large protein-RNA coimmunoprecipitation screenings (Jønson et al., 2007; Bolognani et al., 2010). It appears more likely that SMN, analogous to its function as a chaperone for snRNP biogenesis, is required for the assembly of neuronal mRNA transport complexes (Li et al., 2014). Further studies will be needed to investigate whether HuD and IMP1 overexpression can mitigate SMN-dependent defects in mRNP assembly. One possibility is that HuD and IMP1 overexpression may efficiently compete with other SMN binding partners, leading to a net increase in the number of assembled mRNP complexes and thus enhancing transport and local translation of *GAP43* and possibly other axonal mRNAs.

In conclusion, our study supports a model whereby SMN depletion affects the ability of mRBPs to transport and enhance the local translation of several mRNAs. This suggests that, collectively, their deficiency may contribute to axon degeneration, muscle denervation, and motor neuron death in SMA.

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