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Jeremy Rouanet April 15, 2014

Axonal transport of spinal muscular atrophy (SMA) disease protein survival of motor neuron (SMN) and rescue of axonal defects by the mRNA-binding proteins IMP1 and HuD

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Highest Honors

Neuroscience and Behavioral Biology Program

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Abstract

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By Jeremy P. Rouanet

Spinal muscular atrophy (SMA) is a fatal autosomal recessive genetic disorder that causes motor neuron degeneration and muscle atrophy. It is caused by a mutation or deletion in the survival of motor neuron (*SMN1*) gene and the consequent reduced levels of SMN protein. SMN is ubiquitously expressed, and its best-characterized function is in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). Since SMN is essential for the survival of all cells, it is still unknown why motor neurons are selectively affected in SMA. SMN has been shown to localize to motor neuron axons, and to associate with mRNA-binding proteins, such as IMP1 and HuD, which are decreased in SMA motor neuron axons. This suggests an alternative role for SMN in the regulation of axonal mRNA localization, which is impaired in SMA. In this study, I explored this non-canonical role for SMN by overexpressing IMP1 and HuD in primary cultured spinal motor neurons from a severe SMA mouse model. I observed a rescue of axon length and axonal growth cone protein levels of GAP-43, a locally-translated protein important for axon outgrowth and cytoskeletal maintenance, providing further support for a role of SMN in axonal mRNA metabolism. I further investigated this pathway by adapting the recently-developed split kinesin assay for use in fixed primary spinal motor neurons. This is a promising new assay to potentially identify the motor proteins involved in SMN axonal transport. Finally, I conducted pilot experiments for the future use of γ-secretase modulators to increase axonal transport in murine SMA spinal motor neurons.

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Acknowledgements

I would like to thank Dr. Wilfried Rossoll, Dr. Gary J. Bassell and members of the Bassell lab, especially Paul G. Donlin-Asp and Dr. Claudia Fallini for their assistance and guidance with this project. This work was supported by Families of SMA (FSMA), the Muscular Dystrophy Association (MDA) and the Weisman Family Foundation.

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Introduction

Spinal muscular atrophy (SMA) is the second most common autosomal recessive genetic disorder and the most common genetic cause of infantile death (Wirth et al., 2006). SMA is characterized by the degeneration of lower motor neurons resulting in denervation and muscle atrophy. SMA is classified into types 0-IV, which are determined by the age of onset and the severity of symptoms. Types I is the most common form, in which infants develop hypotonia, are unable to sit or walk, and usually die from respiratory failure within the first two years of life (Wirth et al., 2006).

SMA is caused by mutations or deletions in the *survival of motor neuron 1* (*SMN1*) gene, which encodes for SMN protein (Lefebvre et al., 1995). SMN protein is ubiquitously expressed and has a well-studied role in spliceosomal biogenesis (reviewed in Kolb et al., 2007). Humans carry a duplicated SMN encoding gene, *SMN2*, which is present in a variable number of copies (2-8). However, about 90% of *SMN2* mRNA excludes exon 7 due to a single nucleotide change in a splice site and results in expression of a truncated and unstable SMN protein termed SMNΔ7. Therefore, SMA is caused by a severe reduction in SMN protein levels as opposed to a complete loss (Lorson et al., 1999). The severity of SMA is inversely proportional to the amount of full-length SMN protein produced by *SMN2* (Wirth et al., 2006).

The roles of SMN

The canonical function of SMN is the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) where it acts in a multi-protein complex containing Gemins 2-8 and UNR-interacting protein (UNRIP). This suggests a splicing defect in SMA of certain pre-mRNAs that require snRNPs formed by the SMN complex. Recent work has

suggested the minor spliceosome pathway may contribute significantly to SMA pathology (Gabanella et al., 2007). However, a clear link between splicing defects and the specific neuropathology of spinal motor neurons in SMA has not been found (Burghes and Beattie 2009). Since SMN is ubiquitous and necessary for the survival of all cells, it remains unclear why low levels of SMN affect spinal motor neurons most severely. It has been shown that *β-actin* mRNA and poly(A) mRNAs are reduced at the growth cones of SMA motor neurons (Rossoll et al., 2003; Fallini et al., 2011) and to a lesser extent also in SMA sensory neurons (Jablonka et al., 2006). This suggests that SMN may also function in the assembly of messenger ribonucleoproteins (mRNPs) in the cytoplasm, which are necessary for the stability, localization, and translation of specific mRNAs, such as *β-actin* mRNA. SMN has been shown to associate with a multitude of mRNA-binding proteins (mRBPs) including hnRNP R, hnRNP Q, FMRP, HuD and IMP1 and is localized in dendrites, axons and growth cones of motor neurons (reviewed in Fallini et al., 2012). Therefore, SMN may have an additional function in the assembly, stability and/or transport of mRNPs for local translation of specific mRNAs in distinct subcellular compartments. Defects in these processes may specifically affect spinal motor neurons due to their large size, highly polarized morphology, and unique synaptic terminals. SMA pathology starts at the neuromuscular junctions (NMJs) and has been described as a NMJ synaptopathy where proximal muscles are more affected than distal ones (Kariya et al. 2008).

Axonal defects in SMA animal models

The generation of several animal models for SMA has allowed researchers to study the consequences of SMN deficiency. Knocking out the single SMN-encoding gene *Smn* in

mice results in death during early embryonic development, which indicates that SMN is essential for the survival of all cells through its role in snRNP biogenesis (Schrank et al., 1997). However, inserting a human *SMN2* transgene into an *Smn* null background produces a severe SMA mouse model, characterized by muscle denervation, paralysis, and death shortly after birth (Monani et al., 2000). SMN-deficient motor neurons show a multitude of synaptic defects, including decreased neurotransmitter release, accumulation of phosphorylated neurofilament protein, and immature neuromuscular junctions (reviewed in Fallini et al., 2012). Importantly, axon degeneration precedes the loss of spinal motor neurons (Cifuentes-Diaz et al., 2002). Although embryonic development appears to be unaffected, cultured embryonic motor neurons from the severe SMA mouse model have shorter axons and smaller growth cones compared to wild-type littermate counterparts (Rossoll et al., 2003). Reduced *β-actin* mRNA and protein in the axons and growth cones of SMA motor neurons may explain their altered axonal morphology (Rossoll et al., 2003). β-actin is necessary for cytoskeletal structure, maintenance and motility of neuronal processes, growth cone turning and axonal branching (Zhang et al., 2001, Donnelly et al., 2013). Studies inspired by these findings have shown the mislocalization of several mRNA-binding proteins (mRBPs) in SMA motor neurons, which facilitate axonal localization and translation of various mRNAs, including *β-actin* mRNA (Fallini et al., 2011; 2014; Donnelly et al., 2011).

Recent data from our lab indicate the additional mislocalization of *Gap43* mRNA and protein in SMA motor neurons (Fallini et al., in preparation). *Gap43* encodes the nervous tissue-specific growth-associated protein 43 (GAP-43), which is localized to neuron growth cones and is crucial for proper axonal outgrowth, pathfinding, and

regeneration (reviewed in Denny, 2006; Donnelly et al., 2013). Local translation of mRNAs such as *Gap43* and *β-actin* in the axon and growth cone is critical for neuronal cytoskeletal maintenance, axon pathfinding, growth cone turning, branching and growth (Donnelly et al., 2013), and may be an important biological process affected in SMA motor neurons (Fallini et al., 2012).

IMP1 and HuD associate with SMN

The mRBPs insulin-like growth factor 2 mRNA binding protein 1/zipcode-binding protein 1 (IMP1/IGF2BP1/ZBP1) and HuD/ELAVL4 have been shown to form neuronal ribonucleoprotein complexes together (Yoo et al., 2013), which associate with SMN and are actively transported in axons (Akten et al., 2011; Hubers et al., 2011; Fallini et al., 2011; 2014). Both IMP1 and HuD associate with SMN in axonal granules via its protein-interacting Tudor domain (Fallini et al., 2011; 2014). IMP1 and HuD bind, stabilize, and regulate transport and translation of mRNAs containing specific *cis*-acting regulatory elements (Nielsen et al., 1999; reviewed in Bronicki and Jasmin 2013). Most notably, they form a complex with *β-actin* and *Gap43* mRNA and facilitate their transport to the growth cone for local translation (Yoo et al., 2013). Axonally translated *β-actin* and *Gap43* mRNA are required for proper axon outgrowth, maintenance, and branching (Donnelly et al., 2013). Recently, we have shown that axonal localization of not only HuD but also IMP1, is reduced in SMA motor neurons (Fallini et al., 2011; 2014). Reduced axonal localization of *β-actin* mRNA (Rossoll et al., 2003), *Gap43* mRNA (Fallini et al., in preparation), and the mRBPs IMP1 and HuD (Fallini et al., 2011; 2014) caused by low levels of SMN in SMA motor neurons may provide a link between SMN deficiency and the specific neuropathology of SMA. Artificially increasing axonal

localization of mRBPs such as IMP1 and HuD may be therapeutic in SMA, potentially rescuing an underlying defect in mRNA local translation. To test this hypothesis, we have decided to explore this possibility through IMP1 and HuD overexpression experiments in SMA motor neurons.

Active transport of SMN in the axon

It has been shown that SMN colocalizes with IMP1 and HuD in axonal granules (Fallini et al., 2011; 2014) and is actively transported in axons bidirectionally along microtubules at rates of 1 μm/sec, which is consistent with fast axonal transport (Zhang et al., 2003). SMA motor neurons are characterized by reduced axonal populations of mRBPs, mRNAs and downstream proteins responsible for the development and structural integrity of NMJs (reviewed in Fallini et al., 2012; Fallini et al., in preparation).The axonal localization of the mRBPs HuD and IMP1 and their target mRNAs are SMN-dependent, however; the mechanism by which SMN regulates their localization is not known. SMN could aid in the assembly of mRNPs by facilitating mRNA loading onto mRBPs and/or it could stabilize and facilitate anterograde transport of mRNPs in the axon. Several mRBPs have been shown to be transported in RNA granules associated with KIF5 motor proteins (Kanai et al., 2004) and KIF1Bβ (Charalambous et al., 2013). These motor proteins are kinesins, which are a subset of motor proteins that travel along microtubules in an ATP-dependent fashion. The motor proteins responsible for SMN translocation have not yet been determined. This may be due, in part, to the difficulty of studying kinesin-cargo interactions. Current assays to test for kinesin-cargo interactions result in high variability and conflicting conclusions, since interactions may be highly regulated and transient *in vivo* (Jenkins et al., 2012). To solve the "cargo problem" (Terada and

Hirokawa, 2000), i.e. to determine which cargoes are transported by which kinesins, Jenkins et al. (2012) developed the “split kinesin” assay to effectively screen candidate kinesins for association with a fluorescently-tagged cargo protein population. It is based on a library of "split kinesins" consisting of a conserved kinesin motor domain and a series of variable cargo-binding kinesin tail domains that can be assembled by chemical dimerization (Jenkins et al., 2012). For this study, I have established this assay for use in motor neurons in order to determine the kinesins associated with SMN.

Goals

The aim of this study is two-fold. First, we would like to see if delivery of IMP1 or HuD can rescue GAP-43 protein levels in SMA motor neuron growth cones. IMP1 and HuD protein levels are reduced in SMA axons due to low levels of SMN. Replenishment of IMP1 and HuD levels through cDNA overexpression may increase axonal localization of *Gap43* mRNA, which in turn may rescue the GAP-43 protein population in axonal growth cones. Second, we would like to determine the kinesins that associate with SMN utilizing the split kinesin assay developed by Jenkins et al. (2012). SMN may have an important role in the assembly of mRNPs in the cytoplasm or as a chaperone molecule for the stabilization and axonal transport of mRNPs to the growth cone. Elucidating the kinesin motor proteins responsible for SMN axonal transport may provide additional targets for novel therapeutics. We hypothesize that IMP1 and HuD overexpression in primary cultured SMA motor neurons will result in a rescue of axon length and GAP-43 protein levels in axonal growth cones and that the split kinesin assay can be applied to fixed motor neurons to identify the motor proteins involved in SMN axonal transport.

In addition, I have performed pilot experiments to test the effects of three small-

molecule drugs on the axonal transport of SMN and associated mRNA-binding proteins and examine downstream effects such as axon length. Several experiments indicate the use of γ-secretase modulators, originally designed for use in Alzheimer's disease, as therapeutic in an SMA zebrafish model (Gassman et al., 2013). These small-molecule drugs alter the cleavage of amyloid precursor protein (APP) through modulation of γ-secretase, however; their therapeutic capability in kinesin-deficient *Drosophila* suggests a function facilitating axonal transport (Gassman et al., 2013). These compounds have been provided by our collaborator Dr. Manfredi from Sfida BioLogic, Inc. Here, I optimize the use of these drugs for testing in motor neurons derived from an SMA mouse model, in order to investigate the effects of increased axonal transport in SMA.

Materials and Methods

SMA mouse model

Mice of the severe SMA mouse model FVB.Cg-Tg(SMN2)89Ahmb Smn^{tm1Msd}/J (Jackson Laboratory Stock # 005024) are homozygous for the human *SMN2* transgene and heterozygous for the targeted *Smn* mutation. 25% of the offspring are homozygous for the targeted mutant Smn1 allele and exhibit symptoms and neuropathology similar to patients afflicted with SMA type I (Monani et al., 2000). These severe SMA model mice die around birth.

Primary motor neuron cell culture and transfection

Spinal motor neurons were isolated from E12.5 mouse embryos, plated on poly-ornithine/laminin coated cover slips and cultured as described (Fallini et al., 2010). Cells were transfected using paramagnetic nanobeads (NeuroMag, OZ Biosciences) as previously described (Fallini et al., 2010).

Immunocytochemistry

Coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA-PBS). Immunostaining for myc (DSHB; 1:500) and SMN (Transduction Labs; 1:1000) or GAP-43 (Abcam; 1:500) was performed overnight at 4℃. Fluorescent secondary antibodies (Jackson Labs; 1:1000) were incubated with the coverslips for 1 hour at room temperature, which were subsequently mounted with mounting medium (prolong gold, Invitrogen) onto glass slides for imaging.

Image acquisition and analysis

Transfected motor neurons were imaged using a wide-field fluorescence microscope (Nikon) and a high-resolution CCD camera (Photometrics). 3D images were deconvolved with Autoquant (Media Cybernetics) to remove out-of-focus signal. Using the Imaris suite (Bitplane), 3D surfaces were constructed manually to include the morphological structure-of-interest (cell body, proximal axon, or growth cone). Proximal axons were defined as the initial 70μm of the longest process measured 20μm from the cell body. Axonal growth cones were defined as 10 μm from the tip of the longest axonal branch, including filopodia. Thresholds for each fluorescence channel were set within the surfaces to create a 3D mask of fluorescence signal which excluded background/null signal. Threshold values remained constant within each experiment. Fluorescence intensity sum was calculated within the thresholded mask and normalized to the 3D structure volume. Figure 1 details this process.

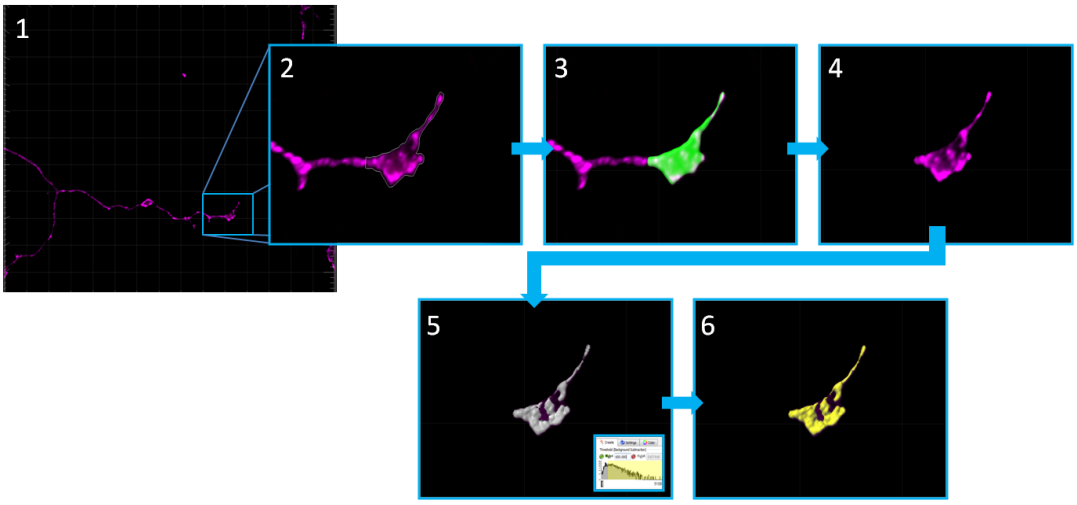

Figure 1. Image analysis of growth cone fluorescence intensity using the Imaris suite. Deconvolved 3D images of axonal growth cones are uploaded to Imaris (1). An area defining the region of the growth cone, 10 µm from the tip of the longest axonal branch is drawn (2) and a 3D surface is constructed (3). A mask within this surface for the channel-of-interest is created (4). Thresholding the fluorescence signal from this mask excludes background fluorescence (5) and another surface, contoured to the thresholded fluorescence signal is created (6), from which fluorescence intensity sum is quantified. This value is normalized to the volume of the structural surface in (3).

Split kinesin assay

Primary motor neurons were co-transfected with expression plasmids for SMN-mGFP, one of eight kinesin cargo domains fused to the FKBP12–rapamycin-binding (FRB) domain, and the motor domain of KIF5C fused to the FKBP domain. All kinesin plasmid constructs were kindly provided by the Banker lab (Fig. 2). Two hours before fixation with 4% PFA-PBS, a rapamycin analogue linker drug (AP21967, Clontech) was added. This cell-permeable ligand induces dimerization of the FRB domain with the FKBP domain (Belshaw et al., 1996) situated on the kinesin tail domains and KIF5C motor domain, respectively (Fig. 3). The motor domain of KIF5C transports the tethered kinesin

tail domain and any bound cargo efficiently along the axon towards the growth cone (Jacobson et al., 2006; Verhey et al., 2011, Jenkins et al., 2012). Axonal SMN protein levels were quantified through high resolution fluorescent microscopy and image analysis software.

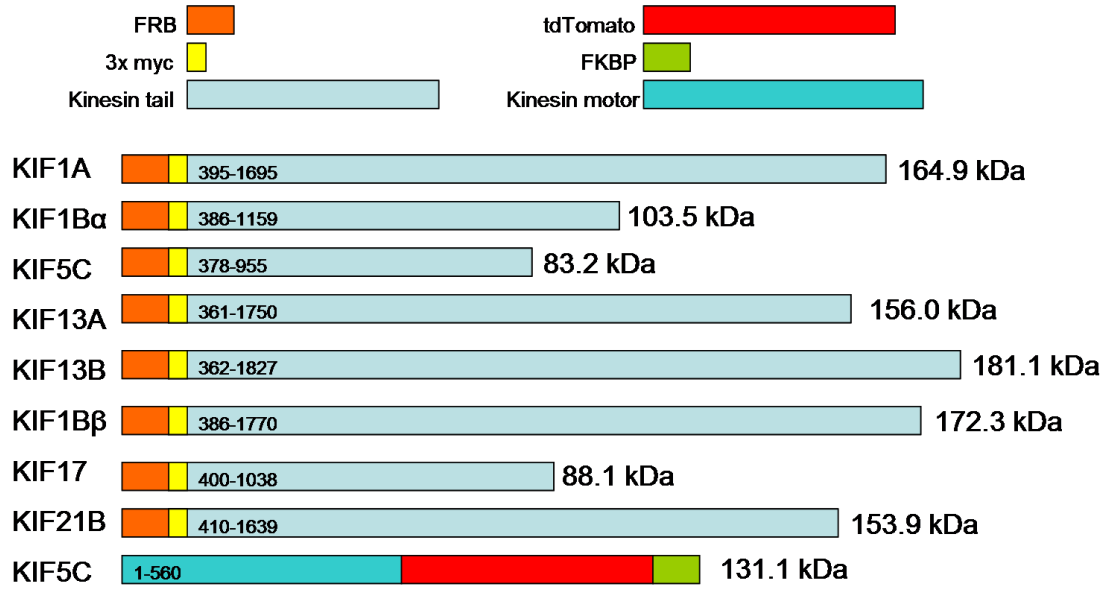

Figure 2. Split kinesin constructs. Eight kinesin tail domains fused to 3xmyc and an FRB domain, in addition to a KIF5C motor domain fused to an FKBP domain and tdTomato (Jenkins et al., 2012), were obtained from the Banker lab. Number ranges indicate amino acids in split kinesin domains.

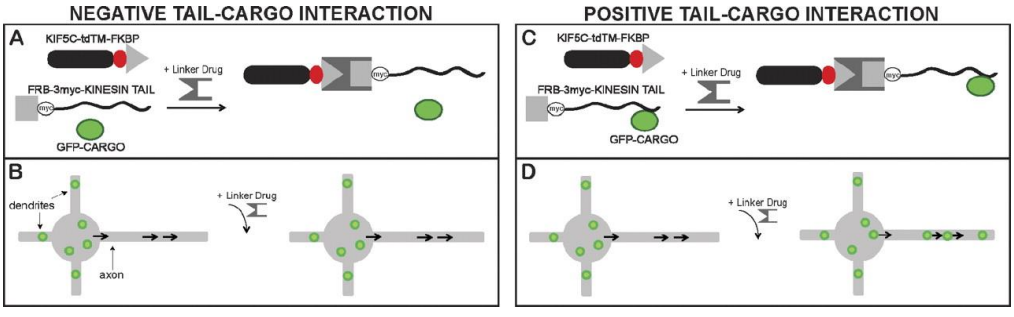

Figure 3. Schematic diagram of the split kinesin assay. Three constructs are expressed together: a cargo protein labeled with GFP, a KIF5C kinesin motor domain tagged with red fluorescent protein tdTomato and fused to FKBP (KIF5C^{559}-tdTM-FKBP), and a

myc-tagged kinesin tail domain fused to FRB. Only if the cargo-binding kinesin tail domain binds its endogenous cargo vesicle (C and D), addition of the linker drug will stimulate axonal transport of the cargo (from Jenkins et al., 2012).

Immunoprecipitation (IP) and western blot analysis

A mouse neuroblastoma cell line, Neuro2a, was transfected with expression plasmids for the KIF5C tail and motor domains. Cells were collected and lysed with lysis buffer containing 1% NP-40 and protease inhibitors (Roche). 50μL protein A agarose beads and 2 μg myc antibodies or control IgGs were incubated overnight at 4˚C with the protein extract. IP pellets were extensively washed, run on a 10% poly-acrylamide gel, and blotted on a nitrocellulose membrane in transfer buffer (25mM Tris, 192mM glycine, 10% methanol, 0.01% SDS). Anti-RFP (1:1000; GeneCopoeia) and myc (1:1000; DSHB) antibodies were used to hybridize the membrane overnight, followed by incubation with fluorescence labeled anti-mouse secondary antibodies and scanning of the blot on a fluorescence imaging system (Odyssey, LiCOR).

Drug treatment

Primary motor neurons were grown for 48 hours *in vitro*. Three drugs obtained from *Sfida*, Inc, dissolved in DMSO and a DMSO vehicle control were added to separate cultures at final concentrations of 0.1μM, 1μM and 10μM 5 hours before fixation or 10μM for periods of 12 hours, 24 hours, 48 hours and 72 hours before fixation. After fixation with 4% PFA-PBS, cells were prepared for immunocytochemistry, imaging and image analysis.

Axon length analysis

Images of GFP-transfected primary motor neurons were acquired using a wide-field

fluorescence microscope and 10x objective (see above) and assembled with Photoshop (Adobe). Length of the longest axonal branch from the cell body to the axon terminus was measured using the NeuronJ plugin for Image J (Meijering et al., 2004).

Statistical analysis

Mean and standard error of the mean (SEM) were used as a measure of central tendency and deviation from the mean, respectively. In all circumstances, "n" refers to number of mouse litters tested. Ordinary one-way ANOVA was used to test for differences in GAP-43 protein levels in the growth cone among the groups. Kolmogorov-Smirnov test was used to assess differences in axon length.

Results

1) Rescue of axonal defects in SMA motor neurons via IMP1 and HuD overexpression

IMP1 and HuD have well-documented roles in the localization of mRNAs necessary for cytoskeletal dynamics, a feature especially critical for the maintenance of highly polarized cells such as spinal motor neurons. Axonal reduction of IMP1 and HuD in SMA motor neurons may contribute to their structural shortcomings. To test this, we asked whether delivery of IMP1 or HuD could rescue axonal defects found in cultured SMA motor neurons.

IMP1 and HuD overexpression rescues axon length defect in SMA motor neurons

First, I tested the effects of IMP1 and HuD overexpression on axon length of SMA motor neurons. Isolated wild-type (WT) and SMA motor neurons were grown in culture for 48 hours, transfected with GFP, IMP1-GFP, HuD-GFP or SMN-GFP and further incubated for an additional 48 hours. Axon length was measured for the longest axonal branches

(Fig 4). Compared to wild-type axons, SMA axons were shorter, as expected (Rossoll et al., 2003). However, overexpression of SMN, HuD, and IMP1 in SMA motor neurons shifts axon length distribution to values similar to those seen of wild-type axons, therefore rescuing the axon length defect seen in SMA motor neurons (Fig 4B). The partial rescue via SMN overexpression may be due to the transfection method and timing used in this experiment, requiring transfection 2 days after plating.

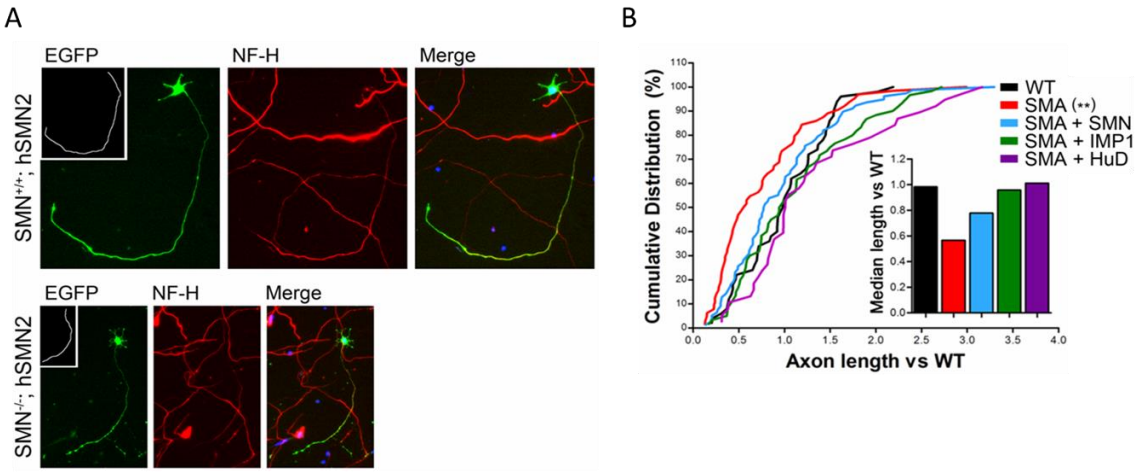

Figure 4. Overexpression of IMP1 and HuD rescues axon length defect in SMA motor neurons. (A) Representative images of WT (top) and SMA (bottom) motor neurons with axon traces (white, top left corners). (B) Normalized cumulative distribution and bar graph of axon lengths from WT-GFP (black), SMA-GFP (red), and SMA motor neurons overexpressing SMN (blue), IMP1 (green), or HuD (purple). Curves shifted towards the left indicate higher frequency of shorter axons compared to WT (Kolmogorov-Smirnov test; ** = $p < .01$; n=3; number of cells >30 for all conditions).

Overexpression of IMP1 and HuD rescues GAP-43 protein levels in SMA motor neuron growth cones

To further investigate the molecular mechanisms possibly mediating rescue of the SMN-dependent axon outgrowth defect via HuD and IMP1 overexpression, we decided to

study their effects on GAP-43 protein levels in the growth cone. GAP-43 is a neuronal growth cone-localized protein important for mediating axonal outgrowth, pathfinding, synaptic plasticity and regeneration (Denny 2006, Donnelly et al., 2013). Recent data from our lab shows GAP-43 protein and mRNA are mislocalized in SMA motor neurons (Fallini et al., in preparation). Importantly, it has been shown that the specific translation of axonally-localized *Gap43* mRNA mediates axonal outgrowth (Donnelly et al., 2013). HuD stabilizes *Gap43* mRNA by binding to an AU-rich regulatory element (ARE) on its 3'-UTR. IMP1 has been shown to be implicated in *Gap43* mRNA localization as well (Donnelly et al., 2011). Thus, recovery of GAP-43 protein expression may be a plausible explanation for the rescue of the axon length defect by overexpression of IMP1 and HuD in SMA motor neurons. SMA and WT motor neurons were isolated and prepared as done previously. GFP, HuD-GFP, or IMP1-GFP was magnetofected and allowed to express for 48 hours. Cells were fixed and immunocytochemistry was used to boost GFP signal to accurately detect transfected cells, fluorescently label GAP-43 (Fig. 5A) and to detect axons with anti-neurofilament staining. Z-stacks of axonal growth cones from positively transfected motor neurons were acquired by a wide-field fluorescence microscope (see Materials and Methods) and growth cone GAP-43 fluorescence intensity was measured by creating a 3D mask of the growth cone using software and quantifying the sum of fluorescence intensity within the mask, normalizing to mask volume (Fig. 1). In Fig. 5B, average values are compared to WT. SMA motor neurons had significantly less GAP-43 protein in the growth cone, while IMP1- and HuD-overexpressed SMA motor neurons showed a recovery of GAP-43 protein expression in the growth cone. Thus, we concluded delivery of HuD and IMP1 rescues a GAP-43 protein localization defect in

SMA motor neurons, which in turn may explain the recovery of axon length, given the function of GAP-43 in axon outgrowth and pathfinding (Donnelly et al., 2013).

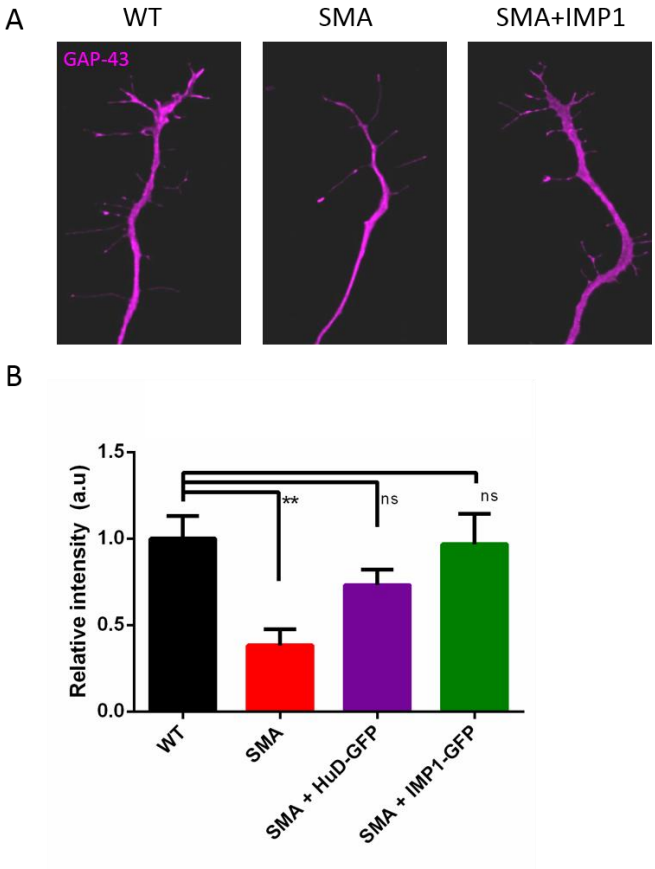

Figure 5. IMP1 and HuD overexpression rescues a defect in GAP-43 protein levels in SMA motor neuron growth cones. (A) Representative images of GAP-43 immunofluorescence (magenta) in growth cones of WT, SMA and SMA+IMP1 motor neurons. (B) Bar graph of average fluorescence intensity normalized to WT. SMA motor neurons had decreased growth cone GAP-43 protein levels compared to WT. Overexpression of HuD or IMP1 resulted in a rescue of this defect (Ordinary one-way ANOVA, ** = $p < .01$; n=3; error bars indicate SEM; number of cells: WT=31, SMA=28, SMA+HuD=29, SMA+IMP1=30).

2) Identification of kinesin motor proteins that mediate the axonal transport of SMN

Because HuD and IMP1 axonal localization is SMN-dependent, and SMN co-localizes

with HuD and IMP1 in the axon, we wanted to further study the axonal transport of SMN. Specifically, we wanted to determine the motor proteins involved in SMN translocation, as this may inform on the composition of SMN-containing granules and their function in the axon, and may also provide additional targets for therapeutic interventions. To do this, I have adapted the split kinesin assay for use in fixed primary motor neurons.

Split kinesin assay

To date, the split kinesin assay has only been used in cultured hippocampal neurons. Kinesin-cargo interactions were mainly determined via live-cell imaging and quantification of axonal anterograde movements of the fluorescent cargo protein population (Jenkins et al., 2012). In the present study, I used the split kinesin assay for the first time in cultured primary motor neurons and assessed interaction by quantifying terminally-accumulated fluorescent protein in a fixed-cell preparation. Several experiments were completed to test the viability of this assay in these conditions, as well as to identify kinesins potentially associated with SMN.

The KIF1A tail domain is transported to axon terminals upon addition of the linker drug

The first aspects to assess whether the assay can be adapted to motor neurons are the localization of the KIF5C motor domain in axon terminals and the performance of the AP21967 linker drug to induce dimerization of the kinesin motor and tail domains, which should increase terminal localization of the kinesin tail due to the specific and efficient localization of the KIF5C motor domain to axon terminals (Jenkins et al., 2012).

For this end, I used a GFP-tagged KIF1A tail construct and quantified green

fluorescence in growth cones as a measure of terminal protein accumulation. After 2 days in culture, motor neurons were co-magnetofected with GFP-KIF1A tail and KIF5C-tdTM motor domains and incubated for an additional 24 hours. Two hours before fixation with 4% PFA-PBS, the linker drug (1μM final concentration) was either added to induce chemical dimerization of the motor and tail domains or omitted. Growth cones were imaged and fluorescence intensity was quantified within 3D masks. Indeed, I observed specific terminal localization of the red fluorescent protein-tagged KIF5C motor domain (Figs. 6A, 7A, 8A). In addition, relative to cells untreated with the linker drug, treated cells displayed a 3-4-fold increase of GFP-KIF1A tail in growth cones (Fig 6B; n=2), indicating successful dimerization and localization to the axon terminus upon treatment. Next, I tested this scenario with the inclusion of a fluorescent protein-tagged cargo.

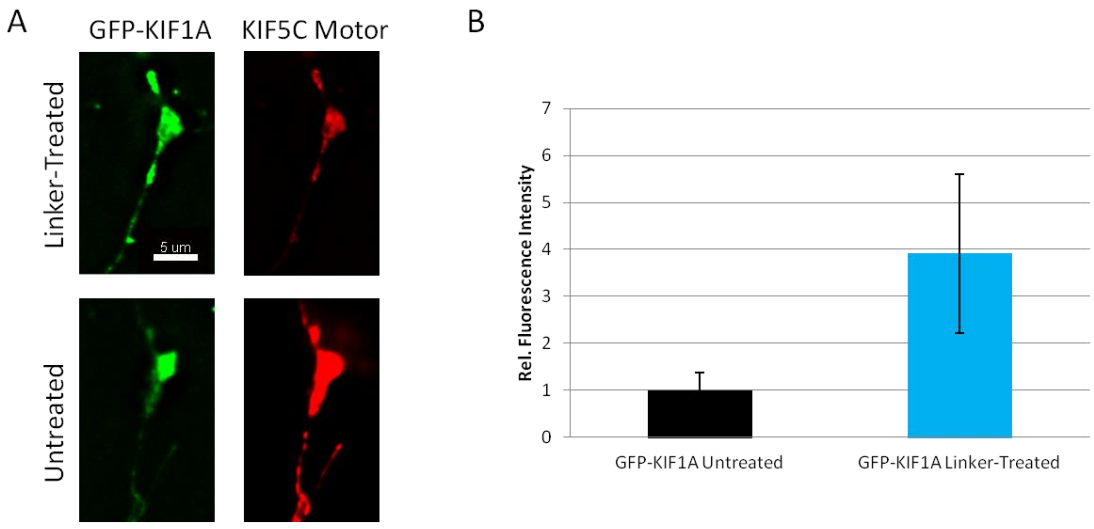

Figure 6. GFP-KIF1A is increased in the growth cones of linker drug-treated primary motor neurons. (A) Representative images of growth cones of denoted treatment conditions. (B) Bar graph of average fluorescence intensity of GFP-KIF1A tail in the growth cone relative to average fluorescence in untreated cells. Addition of the linker drug produced an increase of relative fluorescence intensity by a factor of 3.91,

indicating increased terminal localization of the tail domains due to chemical dimerization with the motor domain (Error bars indicate SEM, n=2; number of cells: Untreated=6, Linker-Treated=6).

Normally dendritically-localized Transferrin receptor is transported to the axonal growth cone via dimerization of myc-KIF13B tail and KIF5C-tdTM motor domains

The previous experiment indicated that the kinesin tail and motor domains can dimerize and translocate successfully to motor neuron growth cones upon exposure to the linker drug and that this localization can be detected in fixed-cell conditions. However, co-expressing a third construct, the fluorescent protein of interest, presented another challenge. Jenkins et al. (2012) reported a novel interaction between the normally dendritically-localized transferrin receptor (TfR) and the KIF13B tail domain through the split kinesin assay and live-cell imaging. Here, I replicate this finding in fixed motor neurons. Like before, motor neurons were isolated and grown in culture for 2 days. At which point, TfR-GFP, 3xmyc-KIF13B tail and KIF5C-tdTM motor constructs were co-magnetofected and allowed to express for 24 hours. Half the cells received treatment of the linker drug 2 hours before fixation. Immunocytochemistry was then performed to detect the myc-epitope-tagged KIF13B tail (αmyc). Growth cones of transfected cells were imaged for fluorescence quantification and results are shown in Figure 7. Upon administration of the linker drug, I observed a 4-5-fold increase of TfR-GFP in the growth cone, and a 3-4-fold increase of the KIF13B tail compared to untreated cells. Of note, the increased localization of the tail domain is consistent with the relative increase measured with GFP-KIF1A. Based on the increase in TfR-GFP at the axonal growth cones of treated cells, I concluded that the KIF13B tail associates with TfR-GFP (as

shown by Jenkins et al., 2012) and both proteins are accumulated at the growth cone by chemical dimerization of the kinesin tail and motor domains. This result provided proof-of-principle that kinesin-cargo interactions can be investigated through the split kinesin assay in fixed motor neurons. Therefore, I next screened several candidate kinesin tails for association with GFP-tagged SMN using this assay.

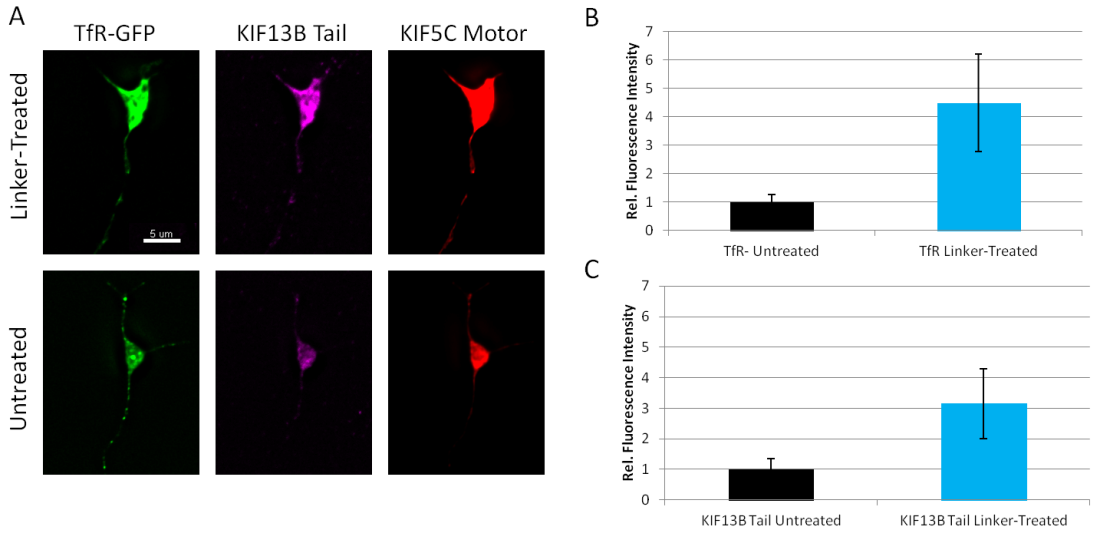

Figure 7. Dendritically-localized TfR is transported to axonal growth cones via chemical dimerization of KIF13B tail and KIF5C motor. (A) Representative images of growth cones from linker-treated (top) and untreated (bottom) cells, showing immunofluorescence for TfR-GFP (left), KIF12B tail (middle) and KIF5C motor (right). (B) Bar graph of average TfR-GFP fluorescence intensity relative to untreated cells. TfR-GFP fluorescence increases 4-5-fold in linker drug-treated cells compared to untreated cells expressing KIF13B tail and KIF5C motor, indicating a potential cargo-tail interaction. (C) Bar graph of average KIF13B tail fluorescence intensity relative to untreated cells. Increased terminal accumulation of the KIF13B tail in linker drug-treated cells indicates successful dimerization of the tail and motor domains, which localizes to axonal growth cones (A-right; Error bars indicate SEM; n=2; number of cells: Untreated=7, Linker-treated=9).

SMN may associate with the KIF21B tail

To test association of SMN with kinesin tail domains, I replicated the conditions for the TfR-GFP and KIF13B tail experiment, substituting in SMN-mGFP as the cargo and other kinesin tail domains. Primary motor neurons were isolated and cultured, magnetofected, treated with the linker drug and prepared for fluorescence microscopy and growth cone fluorescence intensity analysis as before. Figure 8 shows a summary of my findings. Similar to the previous experiments, an increase of KIF21B tail was detected in axonal growth cones of treated motor neurons. SMN-mGFP fluorescence intensity was also increased in treated cells compared to untreated. This suggests an association between SMN-mGFP and the KIF21B tail, which was actively transported to axonal growth cones via chemical dimerization with the KIF5C motor domain. KIF21B is a plus end-directed neuronal kinesin enriched in dendrites with unknown cargoes (Marszalek et al., 1999). Although it has been shown that SMN translocation is consistent with fast motor-dependent transport (Zhang et al., 2003), an association of SMN with a kinesin motor protein, let alone KIF21B, has not yet been shown (see Discussion).

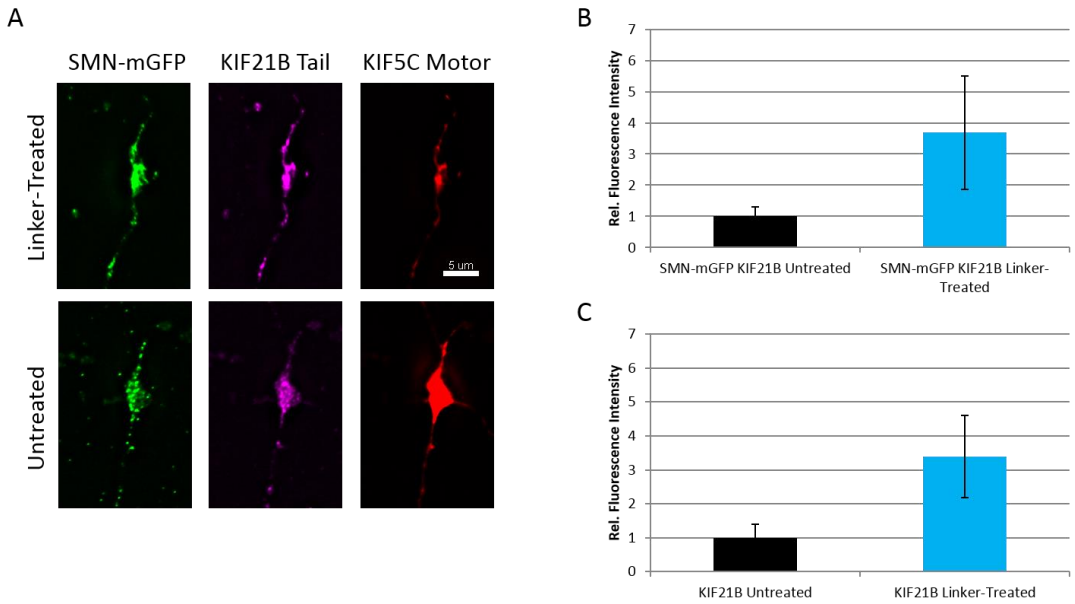

Figure 8. Split kinesin assay reveals possible cargo-tail interaction between KIF21B and SMN-mGFP. (A) Representative images of growth cones showing SMN-mGFP (left), KIF21B tail (middle) and KIF5C motor immunofluorescence in linker drug-treated (top) and untreated motor neurons (bottom). (B) Bar graph showing average growth cone SMN-mGFP immunofluorescence relative to untreated cells. SMN-mGFP is increased in the growth cones of motor neurons expressing KIF21B tail and KIF5C motor when exposed to the linker drug. (C) The KIF21B tail accumulates at the growth cones of linker drug-treated motor neurons confirming dimerization of the tail and motor domains (Error bars indicate SEM; n=3, number of cells: Untreated=17, Linker-treated=10).

Modification to the split kinesin assay is required to test KIF5C association with SMN

Given the association of SMN with mRNA-binding proteins that are present in actively transported RNP granules, SMN may also associate with the motor proteins responsible for their translocation. Of the eight kinesin tail domains provided by the Banker lab, we were especially interested in the potential association of KIF5C with SMN, since KIF5C has been shown to be involved in the anterograde transport of RNP granules in axons (Kanai et al., 2004; Hirokawa et al., 2010). Primary motor neurons were prepared as previously described, except SMN-mGFP and myc-epitope-tagged KIF5C tail constructs were used. Figure 9B-C displays SMN-mGFP and myc-KIF5C immunofluorescence intensity relative to untreated cells. No difference in SMN-mGFP fluorescence was detected between growth cones in linker drug-treated and untreated cells (Fig. 9B). However, the same is true of the KIF5C tail, indicating a possible flaw of the split kinesin assay (Fig. 9C). Possible explanations include failure of the linker drug to induce dimerization of the tail and motor domains, or autodimerization of the domains in absence of the drug. Interestingly, I observed high levels of SMN-mGFP fluorescence in

the growth cones of untreated cells. Figure 9D compares these to the levels detected with the KIF21B tail in experiments that were run in parallel.

To test whether the tail and motor domains are associating in the absence of the drug, I overexpressed both constructs in neuro2a cells and immunoprecipitated the tail domain via its 3xmyc tag (see Materials and Methods). Interestingly, the KIF5C motor domain co-purified with the KIF5C tail domain in cell lysates (Fig. 9E), supporting the theory that these domains may be dimerizing in motor neurons, via homodimerization with either endogenous full length KIF5C, or through the coiled-coil region in their overlapping stalk domain (aa378-560). Thus, further experimentation is necessary to investigate the possible association between KIF5C and SMN, due to the technical shortcomings of using the KIF5C motor domain in the split kinesin assay. Increased levels of SMN-mGFP in axonal growth cones of motor neurons expressing the KIF5C constructs warrant further exploration of KIF5C as a motor protein for SMN axonal transport (Fig. 9D). Modifications to the split kinesin assay may be required to achieve this (see Discussion)

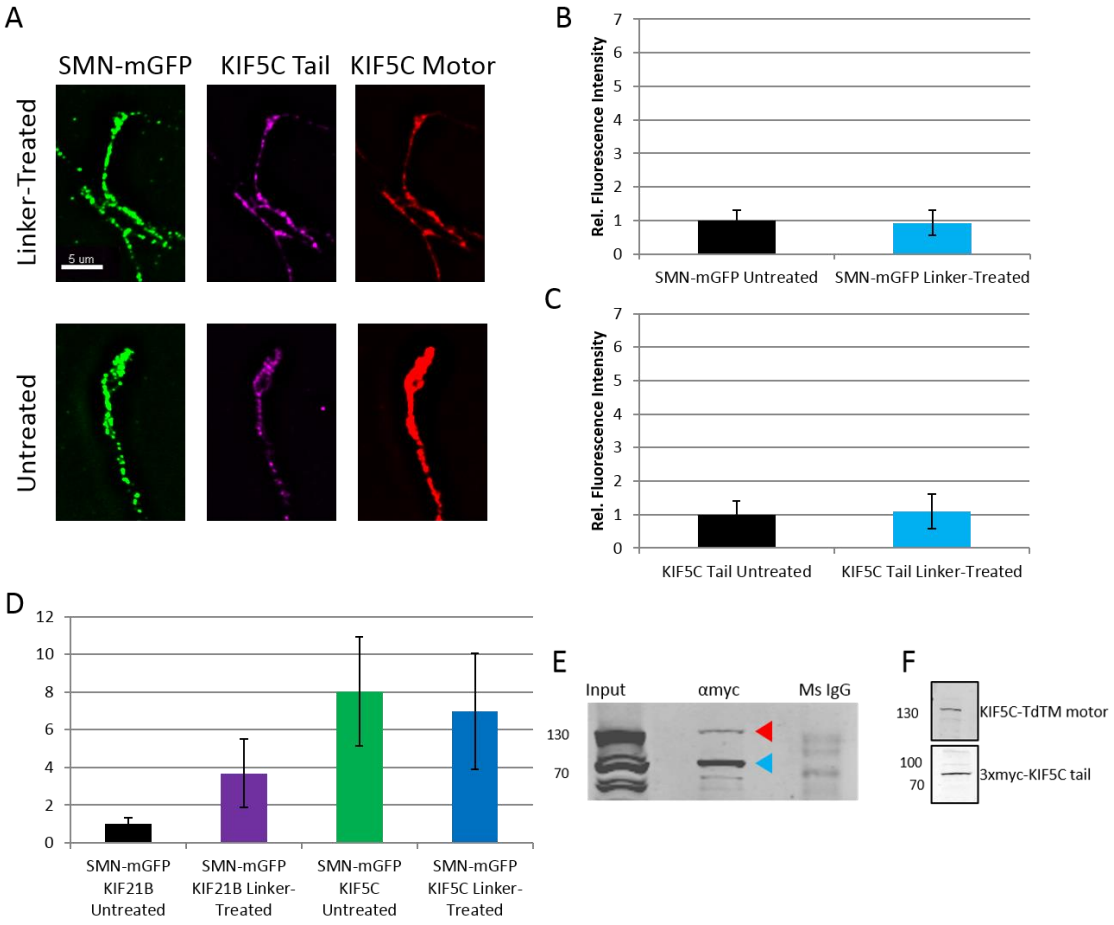

Figure 9. Adapted split kinesin assay is ineffective for probing association of SMN-mGFP and KIF5C. (A) Representative images of linker drug-treated (top) and untreated (bottom) motor neuron growth cones expressing SMN-mGFP (left), KIF5C tail (middle) and KIF5C motor (right). (B) Bar graph showing average fluorescence intensity relative to untreated cells. SMN-mGFP levels do not differ between untreated and linker drug-treated cells. (C) Bar graph showing average growth cone KIF5C tail fluorescence intensity relative to untreated cells. No difference is detected between untreated and linker drug-treated cells, indicating a failure of the split kinesin assay for KIF5C dependent cargoes (Error bars indicate SEM; n=3, number of cells: Untreated=19, Linker-treated=18). (D) Bar graph showing average SMN-mGFP growth cone fluorescence intensity in untreated and linker drug-treated motor neurons expressing the KIF5C constructs and linker drug-treated motor neurons expressing KIF21B tail+KIF5C motor relative to untreated motor neurons expressing KIF21B tail+KIF5C motor.

Independent of the linker drug, there are increased levels of SMN-mGFP in the growth cone in motor neurons expressing the KIF5C constructs. (E) Western blot showing co-immunoprecipitation (αmyc, middle lane) of the KIF5C tail (blue arrow) and KIF5C motor (red arrow) domains in cell lysates of neuro2a cells overexpressing both constructs. The KIF5C tail was immunoprecipitated via its 3xmyc epitope and KIF5C motor was detected via its fused tdTM fluorescent protein (αRFP). Mouse IgG was used as a control (right lane). (F) Representative bands from lysates of neuro2a cells expressing the KIF5C tail and motor domains independently. See Figure 2 for molecular weights.

3) γ-secretase modulators as modifiers of axonal transport in primary motor neurons

Three small-molecule drugs, SBL-154, SBL-185 and SBL-190, were obtained from Dr. Manfredi of Sfida BioLogic, Inc. These drugs are γ-secretase modulators, which modify cleavage of APP by γ-secretase to reduce accumulation of Aβ42, a primary component of amyloid plaque in Alzheimer's disease pathology. Interestingly, these compounds have been shown to be therapeutic in a zebrafish model of SMA, and recent evidence supports a role of these drugs in facilitating axonal transport, suggested by their therapeutic capabilities in kinesin-deficient *Drosophila* (Gassman et al., 2013). We were interested in testing these drugs in motor neurons from an SMA mouse model, and whether they stimulate axonal localization of SMN and its associated mRNA-binding proteins. However, since these drugs have not been tested previously in primary motor neurons from embryonic mouse spinal cords, pilot experiments optimizing their use for these cells *in vitro* were required. I conducted preliminary experiments to determine optimal drug final concentration and incubation time. To determine optimal concentration, I tested the ability of the drugs to stimulate axonal transport at several concentrations. The mRNA-

binding protein staufen was used as a marker for mRNA transport granules (Kanai et al., 2004), and MitoTracker was used to label mitochondria that are actively transported in axons. Based on experiments from Gassman et al. (2013), I selected three concentrations to test (0.1μM, 1μM, and 10μM final concentrations) and treated cultured spinal motor neurons from WT E12.5 mouse embryos for 5h *in vitro* before fixation with 4% PFA-PBS. DMSO was added at matching final concentrations for control conditions. After fixation, staufen was immunofluorescently labeled and coverslips were prepared for fluorescence microscopy and image analysis. I have quantified mitochondria and staufen levels in the proximal axon (initial 70μm from the cell body), and growth cone. Results using 10μM of each drug are displayed in Fig. 10. Final concentrations of 0.1μM and 1μM did not produce an increase of mitochondria or staufen (data not shown), however slight increases were detected in the axon and growth cone for SBL-185 and SBL-190 at 10μM compared to DMSO at the same concentration, indicating 10μM as an optimal final concentration for these drugs to affect axonal transport in this assay.

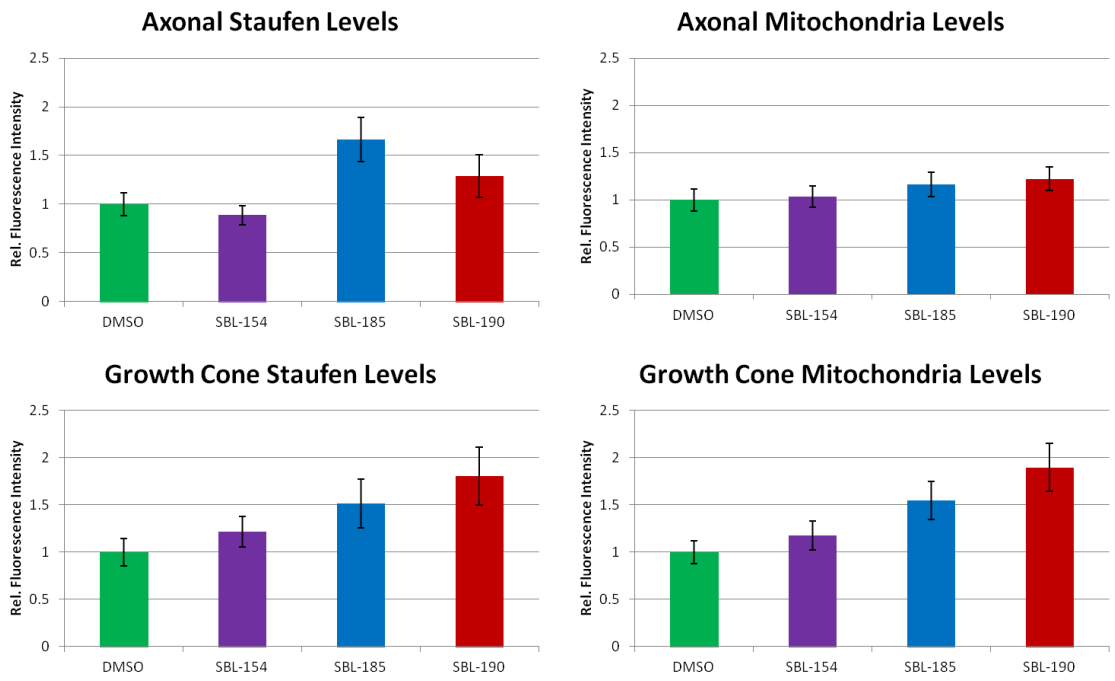

Figure 10. *Sfida* Compounds increase staufen and mitochondria levels in the axons and growth cones of primary spinal motor neurons. (*Top panels*) Axonal staufen (left) and mitochondria (right) average fluorescence intensity in designated drug-treated cells (10μM final concentration) relative to DMSO vehicle-treated cells (n=3, number of cells: DMSO=24, SBL-154=24, SBL-185=26, SBL-190=26). (*Bottom panels*) Staufen (left) and mitochondria (right) levels in the growth cone in drug-treated cells compared to vehicle-treated cells (n=3, number of cells: DMSO=27, SBL-154=23, SBL-185=26, SBL-190=27). Cells were treated for 5h with one of the drugs or DMSO vehicle and assessed for changes in axonal transport. Axonal and growth cone staufen and mitochondria levels are increased in cells treated with SBL-185 and SBL-190 (Error bars indicate SEM).

Next, I tested their effect on axon length at incubation times of 12h, 24h, 48h, and 72h, as Gassman et al. (2013) reported the drugs facilitate axon outgrowth in rat spinal motor neurons. At 24h before fixation, cells were magnetofected with GFP to highlight cell morphology. GFP signal was boosted with immunocytochemistry (αGFP) to make

transfected cells easily detectable at low magnification. Only transfected cells were scored for axon length (longest axonal branch), which was measured using tracings produced in the NeuronJ plugin for ImageJ (Meijering et al., 2004). Results are shown in Fig. 11. Slight increases of axon length were only seen in cells incubated with the drugs for 48h. However, it was noted that increased cell death occurred in drug-treated conditions (data not shown), which may have biased results. Therefore, 10µM final concentration may be optimal to detect changes in axonal transport, but it may be too strong for longer incubation periods. Lower concentrations of these drugs may be required for experiments with long incubation times.

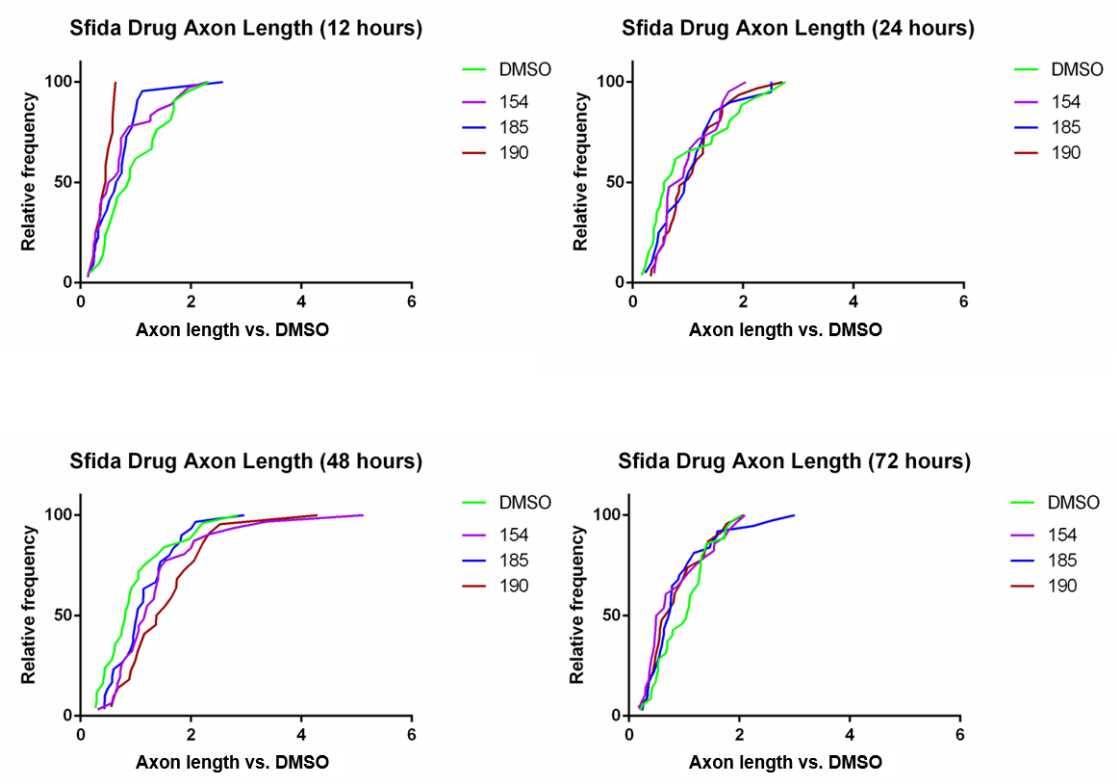

Figure 11. *Sfida* compounds increase axon outgrowth with 48h incubation. Cumulative distributions of axon lengths of motor neurons treated with 10µM SBL-154 (magenta), SBL-185 (blue), SBL-190 (red) or DMSO-vehicle (green) for 12h (top left), 24h (top right), 48h (bottom left), or 72h (bottom right). No detectable difference in axon length was observed with incubation times of 12h, 24h, or 72h. Increased axon outgrowth

in drug-treated cells compared to vehicle-treated cells occurred with 48h incubation (n=3; number of cells > 10 for all conditions).

Discussion

This is the first study to investigate rescue of SMA motor neuron defects through direct restoration of mRNA-binding protein levels and the first to suggest decreased local translation of *Gap43* mRNA may be a direct effect of low SMN levels in SMA. Total or partial recovery of GAP-43 protein in the growth cone can be achieved by overexpression of the mRNA-binding proteins IMP1 or HuD in SMA motor neurons, which also results in recovery of axon outgrowth. This is highly relevant for SMA research, since it suggests that these defects are not related to SMN's canonical role in splicing, but rather an additional role in axonal mRNP delivery. This study is also the first to address kinesin-mediated axonal transport of SMN. I have established the split kinesin assay in fixed motor neurons to identify the motor proteins involved in transport of SMN. In addition, I have conducted pilot experiments for the use of several small-molecule drugs to test the effects of increased axonal transport in SMA motor neurons.

Overexpression of IMP1 and HuD rescues axonal defects in SMA motor neurons. Although SMN has a canonical role in snRNP biogenesis, it has been shown to interact with other RBPs such as IMP1 and HuD. Recent work from our lab demonstrates low levels of SMN result in a reduction of IMP1 and HuD in SMA motor neuron axons (Fallini et al., 2011; 2013). Here I tested the significance of these pathological features by rescuing IMP1 and HuD protein levels through overexpression in SMA motor neurons. I found that IMP1 and HuD overexpression resulted in a rescue of axon length and GAP-

43 protein levels at the growth cones of SMA motor neurons. IMP1 and HuD are involved in the stability and axonal localization of *Gap43* mRNA, which is also mislocalized in SMA (Fallini et al., in preparation). Although currently untested, recovery of IMP1 and HuD levels in SMA presumably leads to a recovery of axonal *Gap43* mRNA localization. Donnelly et al. (2013) report axonally-translated *Gap43* mRNA, as opposed to GAP-43 protein originating from the cell body, results in an increase of axon outgrowth. Thus, increased GAP-43 protein in the growth cone and increased axon outgrowth as a result of IMP1 and HuD overexpression in SMA motor neurons may be explained by recovered axonal localization and translation of *Gap43* mRNA. These data contribute to a larger study from our lab documenting the dysregulation of *Gap43* mRNA in SMA (Fallini et al., in preparation). *Gap43* mRNA mislocalization may be a consequence of impaired axonal localization of IMP1 and HuD in SMA. These overexpression experiments highlight the importance of IMP1 and HuD in motor neuron axons and their contribution to downstream effects such as axon outgrowth and GAP-43 localized translation, which may be a key process affected in SMA spinal motor neurons.

The split kinesin assay is a promising new approach to study motor-dependent transport in primary spinal motor neurons

In order to identify the kinesin motor proteins involved in SMN transport, I have adapted the split kinesin assay for use in fixed motor neurons. Previously, Jenkins et al. (2012) developed the assay for use in live primary hippocampal neurons. Cargo-tail interaction was determined by measuring anterograde axonal transport of the fluorescent cargo protein of interest via live-cell imaging. Increased fluorescent particle movements after treatment with the linker drug indicated an association between the fluorescent cargo

protein and the kinesin tail, which dimerized with the KIF5C motor domain (Jenkins et al., 2012). It was unclear whether I could use a simplified version of this assay in fixed motor neurons, using accumulated protein in the axonal growth cone as a measure for increased anterograde axonal transport. My data show addition of the linker drug results in a specific accumulation of the kinesin tail at axonal growth cones, where the KIF5C motor domain localizes. The normally dendritic TfR-GFP fusion protein also accumulated in the axonal growth cone when I co-expressed it with the KIF13B tail and KIF5C motor domain, replicating one of the cargo-tail interactions reported in hippocampal neurons by Jenkins et al. (2012). They reported changes in anterograde axonal transport in hippocampal cells as early as 15 minutes after addition of the linker drug, which peaked between 20 and 30 minutes. In our experiments, we incubated the linker drug with the cells for 2 hours before fixation, to allow for terminal protein accumulation to occur as a result of increased axonal transport. Other incubation times were not tested in this study. In addition, I used 1μM final concentration of the linker drug as used by Jenkins et al. (2012), although manufacturer's recommendations (Clontech) state this is the maximum nontoxic concentration for cells *in vitro.* Modifications of the incubation time and final concentration of the linker drug may be beneficial to further optimize the split kinesin assay in fixed motor neurons. Because knowledge of kinesin-cargo interactions is limited, one cannot rule out possible associations between a tail and a cargo for negative controls. However, since we do not report a negative result, where the kinesin tail is successfully localized to the axonal growth cone following linker-drug treatment and no increase in axonal growth cone fluorescent cargo protein localization is observed, the specificity of these experiments

needs to be further verified. Additional experiments which show negative results for a kinesin tail-cargo interaction may inform on the specificity of this assay in fixed motor neurons.

KIF21B may be involved in the axonal and/or dendritic transport of SMN

Data from my split kinesin assays in fixed motor neurons suggest KIF21B as a potential motor protein for SMN transport. This is the first study that suggests a potential cargo for KIF21B and a motor protein for SMN transport. KIF21B is a neuronal plus end-directed motor that is expressed in both dendrites and axons, however it is more enriched in dendrites (Marszalek et al., 1999). Not much is known of the function of KIF21B and its cargoes have not yet been identified. Since SMN is localized in the nuclear gems, somas, dendrites, and axons (reviewed in Fallini et al., 2012), it is likely that its transport may be facilitated by several different motor proteins. KIF21B may therefore play a role in SMN dendritic localization. Further studies investigating the relationship between KIF21B and SMN should be conducted to confirm a cargo-tail interaction and explore functional significance.

Modified assays are required to validate KIF5C as a motor protein for SMN axonal transport

In addition to KIF21B, I have also tested KIF5C as a motor protein for axonal transport of SMN. KIF5C has been shown to transport RNP granules containing several of the mRNA-binding proteins associated with SMN in the axon (Kanai et al., 2004) and is specifically enriched in spinal motor neurons of 2-week or older mice, suggesting a role in axon maintenance (Kanai et al., 2000). Considering the need for SMN in NMJ maturation (Kariya et al., 2014) and its co-localization with mRNA-binding proteins in

the axon (Fallini et al., 2011; 2014), KIF5C is a likely candidate for SMN axonal translocation. Interestingly, my experiments showed that there was no change in KIF5C tail axonal growth cone accumulation after linker-drug treatment, suggesting a potential flaw of the split kinesin assay for this kinesin tail domain, as its localization appears to be independent of linker-drug administration. Jenkins et al. (2012) only tested the ability of the KIF1A tail domain to translocate anterogradely along the axon in response to linker-drug treatment using fluorescently-tagged KIF1A (GFP-KIF1A), which was also tested in this study. However, the dimerization and specific axonal transport of the other tails after linker-drug treatment has not been verified. Therefore, it remains to be tested whether the KIF5C tail is specifically transported to axonal growth cones by the KIF5C motor only in response to treatment of the linker drug. It may be possible that the tail domain is dimerizing with endogenous KIF5C, or associating by other means with the KIF5C motor domain in absence of the drug. Immunoprecipitation of the KIF5C tail domain revealed a biochemical association with the KIF5C motor domain in neuro2a cell lysates, where both constructs were expressed. This suggests that non-linker-drug-specific association may be occurring in motor neurons.

Since SMN-mGFP levels in axonal growth cones were increased in motor neurons expressing the KIF5C tail and motor domains compared to cells expressing other tails, the possibility of KIF5C as a motor protein for SMN should be further investigated. However, modifications to the split kinesin assay may be necessary to properly address this potential cargo-motor interaction. As an alternative approach, a different motor domain linked to an FKBP domain, not necessarily of a kinesin family, could be used to dimerize with the KIF5C tail and cause tail/cargo localization to other cell compartments

upon linker-drug exposure. Increased SMN-mGFP localization to the targeted cell compartment would indicate a positive cargo-tail interaction. Using a different motor domain to localize the KIF5C tail domain to other cell compartments would give an additional degree of specificity, since SMN already localizes to the growth cone in normal conditions. In another experiment, the KIF5C tail domain could be expressed independently and endogenous SMN immunofluorescence could be measured. Because the KIF5C tail lacks the motor domain, it may sequester SMN in the cell body, preventing proper axonal localization of SMN in motor neuron axons. Barring possible association of the KIF5C tail with endogenous KIF5C, decreased axonal SMN would indicate sequestration in the cell body due to binding with the KIF5C tail domain.

Pilot experiments show that *Sfida* compounds affect axonal transport in motor neurons

Given the defects of axonal localization of various mRBPs and mRNAs, NMJ maturation, and neurotransmitter release in SMA spinal motor neurons (reviewed in Fallini et al., 2012), the use of axonotrophic agents for SMA therapy appears plausible. Increasing axonal intracellular transport in SMA spinal motor neurons may rescue defects in axonal mRNA localization, which could allow proper axon maintenance and maturation of the neuromuscular junctions (NMJs), and possibly alleviate or prevent the pathological features of SMA. In this study I tested three γ-secretase modulators in WT spinal motor neurons for later application in SMA motor neurons. These small-molecule drugs alter the metabolism of APP by modulating the activity of γ-secretase. APP metabolism has been shown to be tightly linked to intracellular transport, as enzymes that process APP also influence the trafficking and cellular distribution of its proteolytic

fragments (Zhang et al, 2006; Cai et al., 2003; 2006). Gassman et al. (2013) report these drugs rescue kinesin-deficient *Drosophila* larvae, indicating a function of increased axonal intracellular transport. In addition, these compounds were also shown by Gassman et al. (2013) to be therapeutic in SMN-deficient zebrafish embryos. Thus, we were interested in testing these drugs in SMA murine motor neurons, as their therapeutic capabilities may be a result of increased kinesin activity and axonal transport. I conducted pilot experiments in WT motor neurons to determine optimal dosage and incubation times. My results suggest 10μM as an optimal concentration for increasing axonal transport and a 48 hour incubation time as optimal for downstream effects such as axon outgrowth; however a lower concentration may be needed to avoid cytotoxicity. Further experiments are required to investigate effects in axonal localization of SMN and its associated mRBPs, and whether these drugs rescue defects in spinal motor neurons derived from a severe SMA mouse model.

Conclusions

Here, we highlight the importance of mRBP axonal localization in spinal motor neurons through rescue of axonal defects in SMA motor neurons by mRBP overexpression. GAP-43 protein levels in axonal growth cones, which are decreased in SMA, can be rescued by overexpression of SMN-associated mRBPs IMP1 and HuD. Taken together with a deficiency of HuD and IMP1 localization in SMA axons, we conclude a role for SMN in facilitating IMP1 and HuD axonal localization, which results in localized GAP-43 protein expression. Low levels of SMN disrupt this pathway, resulting in mislocalized *Gap43* mRNA, decreased GAP-43 protein levels in the growth cone, and downstream defects in axon outgrowth and cytoskeletal maintenance, which could specifically affect spinal

motor neurons due to their size and highly specialized axon terminals. This pathway may provide additional targets for future therapeutic developments for SMA. Promising therapeutics for SMA include the use of viral vectors carrying *SMN* cDNA to restore SMN protein levels in relevant tissues (reviewed in Monani and De Vivo 2014). In addition, much research has focused on improving the efficiency of *SMN2* expression to produce full-length SMN protein. Antisense oligonucleotides have been developed to alter the splicing of *SMN2* transcripts to increase exon 7 inclusion and subsequently increase SMN protein levels (Monani and De Vivo 2014). Based on our evidence, another intervention may be to increase axonal transport of SMN or its associated mRBPs. In order to further understand the role of SMN in the axon and to provide additional molecular targets for intervention, I adapted the split kinesin assay for use in fixed spinal motor neurons to identify the kinesin motor proteins involved in SMN translocation. In addition, I tested three compounds obtained from *Sfida*, Inc, thought to be therapeutic in SMA through a mechanism of increased intracellular axonal transport, for future use in SMA motor neurons. Understanding mRNA axonal localization dynamics and its therapeutic potential in SMA may one day lead to successful clinical treatments for this currently untreatable disease.

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