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Kathryn R. Williams, Emory University
Damián S. McAninch, Duquesne University
Snezana Stefanovic, Duquesne University
Lei Xing, Emory University
Megan Allen, Emory University
Wenqi Li, Emory University
Yue Feng, Emory University
Mihaela Rita Mihaiescu, Duquesne University
Gary Bassell, Emory University

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hnRNP-Q1 represses nascent axon growth in cortical neurons by inhibiting Gap-43 mRNA translation

Kathryn R. Williamsa, Damian S. McAninchb, Snezana Stefanovicb, Lei Xinga, Megan Allenc, Wenqi Li, Yue Feng, Mihaela Rita Mihaiescub, and Gary J. Bassella

INTRODUCTION

Heterogeneous ribonucleoprotein Q, isoform 1 [hnRNP-Q1 [Mourelatos et al., 2001], Nsap1/Syncrip/hnRNP-Q2 [Harris et al., 1999; Mizutani et al., 2000; Svitkin et al., 2013]] is a ubiquitous mRNA-binding protein that demonstrates high expression in the brain (Mizutani et al., 2000; Rossoll et al., 2002; Xing et al., 2012). It contains two different RNA-binding domains, three RNA recognition motifs, and a single arginine- and glycine-rich region (RGG box) (Mourelatos et al., 2001). hnRNP-Q1 participates in several mRNA processing events, including splicing, editing, transport, translation, and decay (Wigington et al., 2014). Unlike other hnRNP-Q isoforms, hnRNP-Q1 is mainly localized to the cytoplasm, suggesting that functions in mRNA translation, localization, and/or decay regulation are of higher importance (Mourelatos et al., 2001). Supporting this, hnRNP-Q1 has recently been demonstrated to repress RhoA mRNA translation and regulate Cdc42 mRNA localization (Chen et al., 2012; Xing et al., 2012). Given the high expression of hnRNP-Q1 in brain, we predict that hnRNP-Q1 posttranscriptionally regulates the expression of many mRNA targets, which are potentially involved in neuronal development and function.

Gap-43 is a neuronal-specific protein that regulates multiple aspects of neuronal development, plasticity, and regeneration (Denny, 2006). GAP-43 is enriched in axonal growth cones after polarity is stabilized (Denny, 2006). GAP-43 is expressed in brain tissue, suggesting a function in regulating genes critical for neuronal development and function. GAP-43 is a neuronal protein that regulates actin dynamics in growth cones and facilitates axonal growth. Previous studies have identified factors that regulate Gap-43 mRNA stability and localization, but it remains unclear whether Gap-43 mRNA translation is also regulated. Our results reveal that hnRNP-Q1 knockdown increased nascent axon length, total neurite length, and neurite number in mouse embryonic cortical neurons and enhanced Neuro2a cell process extension; these phenotypes were rescued by GAP-43 knockdown. Additionally, we have identified a G-quadruplex structure in the 5′ untranslated region of Gap-43 mRNA that directly interacts with hnRNP-Q1 as a means to inhibit Gap-43 mRNA translation. Therefore hnRNP-Q1–mediated repression of Gap-43 mRNA translation provides an additional mechanism for regulating GAP-43 expression and function and may be critical for neuronal development.

REFERENCES

Mourelatos et al., 2001; Rossoll et al., 2002; Xing et al., 2012. It contains two different RNA-binding domains, three RNA recognition motifs, and a single arginine- and glycine-rich region (RGG box) (Mourelatos et al., 2001). hnRNP-Q1 participates in several mRNA processing events, including splicing, editing, transport, translation, and decay (Wigington et al., 2014). Unlike other hnRNP-Q isoforms, hnRNP-Q1 is mainly localized to the cytoplasm, suggesting that functions in mRNA translation, localization, and/or decay regulation are of higher importance (Mourelatos et al., 2001). Supporting this, hnRNP-Q1 has recently been demonstrated to repress RhoA mRNA translation and regulate Cdc42 mRNA localization (Chen et al., 2012; Xing et al., 2012). Given the high expression of hnRNP-Q1 in brain, we predict that hnRNP-Q1 posttranscriptionally regulates the expression of many mRNA targets, which are potentially involved in neuronal development and function.

Gap-43 is a neuronal-specific protein that regulates multiple aspects of neuronal development, plasticity, and regeneration (Denny, 2006). GAP-43 is enriched in axonal growth cones after polarity is
Elevated GAP-43 expression in hnRNP-Q1–deficient N2a cells and cortical neurons

We first sought to determine whether GAP-43 protein levels were affected by hnRNP-Q1 knockdown, because GAP-43 is an important neuronal protein and hnRNP-Q1 is highly expressed in the brain. N2a cells were transfected with hnRNP-Q1 small interfering RNAs (siRNAs) targeting sequences in the 3′ untranslated region (3′-UTR) that are not present in other hnRNP-Q isomorphs. Scrambled (Scr) siRNA was used as a control. Immunoblot analysis of cell lysates 72 h after transfection revealed that hnRNP-Q1 can be efficiently depleted (siRNA 1 = 0.31-fold, siRNA 2 = 0.44-fold, and siRNA 3 = 0.67-fold; Figure 1A). Interestingly, GAP-43 protein levels were increased according to the degree of hnRNP-Q1 depletion (siRNA 1 = 2.99-fold, siRNA 2 = 2.30-fold, and siRNA 3 = 1.72-fold; Figure 1A). The levels of the highly homologous proteins hnRNPR and hnRNP-Q3 and γ-actin protein were not significantly affected (Figure 1A). hnRNP-Q1 siRNA 1 was used for the remainder of the experiments, because it demonstrated the greatest knockdown. Quantitative real time-PCR (qRT-PCR) was performed to determine whether hnRNP-Q1 knockdown affects Gap-43 mRNA levels. We found that neither Gap-43 nor γ-actin mRNA levels were significantly altered upon hnRNP-Q1 knockdown (1.02-fold and 1.05-fold, respectively; Figure 1B) suggesting that hnRNP-Q1 may regulate GAP-43 expression through a translational mechanism.

Primary cortical neurons were also assessed to determine whether hnRNP-Q1 depletion increases GAP-43 protein expression at the subcellular level. Neurons were electroporated with hnRNP-Q1 #1 or Scr siRNA and Lifeact–green fluorescent protein (GFP) immediately following the dissection and were fixed after 28.5 h in culture. siRNA was used instead of short hairpin RNA (shRNA) due to higher transfection efficiencies and quicker knockdown times, which are required to assess incipient neuron phenotypes. The neurons were then processed for immunofluorescence with GAP-43 and hnRNP-Q1 antibodies, and transfected cells were selected by GFP signal (Figure 1, C and D). The specificity of the hnRNP-Q1 antibody was verified by immunoblotting N2a cell lysates after overexpressing EGFP-tagged hnRNP-Q1 for ~16 h (Supplemental Figure 1A). Because GAP-43 is enriched in axonal growth cones after polarity is established, we quantified GAP-43 protein levels in both the cell bodies and nascent axons of these incipient neurons. hnRNP-Q1 knockdown increased GAP-43 protein levels on average by 1.36-fold in cell bodies and by 1.56-fold in nascent axons (~1.72-fold; Figure 1, E and F). Plotting hnRNP-Q1 protein levels against GAP-43 protein levels for each cell supports an inverse correlation between hnRNP-Q1 and GAP-43 protein levels (Supplemental Figure 1B). hnRNP-Q1 knockdown also did not reduce the ratio of GAP-43 levels in nascent axon/cell body, suggesting that hnRNP-Q1 is not required for GAP-43 protein enrichment in the nascent axon and growth cone (Figure 1G). In fact, the nascent axon/cell body ratio was actually significantly increased upon hnRNP-Q1 knockdown (0.73–0.83, 1.14-fold) suggesting that hnRNP-Q1 negatively regulates GAP-43 levels within the axon (Figure 1G).

An inverse correlation between hnRNP-Q1 and GAP-43 protein levels would support our model that hnRNP-Q1 negatively regulates GAP-43 protein expression. Thus we investigated the expression profiles of hnRNP-Q1 and GAP-43 in cultured neurons. High-density primary cortical neurons were cultured for 0–21 d, and cell lysates were collected every third day and immunoblotted for hnRNP-Q1 and GAP-43. The expression profiles from the time course suggest that hnRNP-Q1 and GAP-43 are both developmentally regulated in primary cortical neurons and that these proteins have opposite patterns. hnRNP-Q1 protein levels decreased over...
FIGURE 1: Increased GAP-43 protein expression upon hnRNP-Q1 knockdown. (A) GAP-43 and γ-actin protein levels were assessed by immunoblot in N2a cell lysates 72 h after hnRNP-Q1 #1, hnRNP-Q1 #2, or Scr siRNA transfection. \( n = 6 \), one-way analysis of variance (ANOVA), Dunnett’s posthoc, \( p \) values: Scr vs. Q1 #1, \( p = 0.3897 \); Scr vs. Q1 #2, \( p = 0.2057 \); Scr vs. Q1 #3, \( p = 0.1801 \); hnRNP-Q3 \( p \) values: Scr vs. Q1 #1, \( p = 0.8869 \); Scr vs. Q1 #2, \( p = 0.4025 \); Scr vs. Q1 #3, \( p = 0.0846 \); hnRNP-Q1 \( p \) values: Scr vs. Q1 #1, \( p < 0.0001 \); Scr vs. Q1 #2, \( p < 0.0001 \); Scr vs. Q1 #3, \( p = 0.0163 \); γ-actin \( p \) values: Scr vs. Q1 #1, \( p = 0.8493 \); Scr vs. Q1 #2, \( p = 0.3335 \); Scr vs. Q1 #3, \( p = 0.9995 \). (B) Gap-43 and γ-actin mRNA levels were assessed by qRT-PCR in N2a cell lysates 72 h after hnRNP-Q1 #1 or Scr siRNA transfection. \( n = 6 \), one-sample \( t \) test, \( p \) values: Gap-43, \( p = 0.6415 \); γ-actin, \( p = 0.8956 \). (C-G) Primary cortical neurons were transfected with hnRNP-Q1 #1 or Scr siRNA + Lifeact-GFP by nucleofection and cultured for 28.5 h. GAP-43 and hnRNP-Q1 were detected by immunofluorescence, and GFP-positive cells were imaged. (C) Representative images with inset heat maps and (D) enlarged views of the nascent axon with a GAP-43 heat map (white boxes in C). Scale bars: 10 µm. Quantification of GAP-43 and hnRNP-Q1 signal intensity in (E) cell bodies and (F) the nascent axon. \( n = 6 \), Scr: 198 neurons and Q1: 178 neurons from six independent experiments, one-sample \( t \) test, cell body \( p \) values: hnRNP-Q1, \( p < 0.0001 \); GAP-43, \( p = 0.0002 \); nascent axon \( p \) values: hnRNP-Q1, \( p = 0.2044 \); GAP-43, \( p < 0.0001 \). (G) Ratio of nascent axon/cell body GAP-43 protein levels. \( n = 6 \), Scr: 198 neurons and Q1: 178 neurons from six independent experiments, one-sample \( t \) test, \( p \) value = 0.0002.
protein levels due to hnRNP-Q1 knockdown affect neurite length. Therefore we next determined whether elevated GAP-43 levels of GAP-43 in primary cortical neurons (Supplemental Figure 1C). Additionally, GAP-43 and the mRNA-binding protein HuD, a positive regulator of GAP-43 expression, have previously been demonstrated to be enriched in Ammon's horn as compared with the dentate gyrus of the hippocampus in vivo (Clayton et al., 1998; Namgung and Routenberg, 2000). Therefore we assessed whether hnRNP-Q1, a proposed negative regulator of GAP-43 expression, demonstrated the opposite pattern of expression. The two regions of the hippocampus were dissected from P30 wild-type mice (Hagihara et al., 2009), and the levels of HuD, Gap-43, and hnRNP-Q1 mRNAs were quantified by qRT-PCR due to an inadequate amount of tissue for immunoblotting. As expected, HuD and Gap-43 mRNAs were enriched in Ammon's horn (2.54-fold and 1.65-fold, respectively; Supplemental Figure 1D). However, hnRNP-Q1 mRNA was not enriched in the Ammon's horn and is potentially more enriched in the dentate gyrus, suggesting that hnRNP-Q1 expression was not enriched in the Ammon's horn and is potentially more enriched in the dentate gyrus, suggesting removal of GAP-43 repression by hnRNP-Q1 knockdown may contribute to GAP-43 protein expression in vivo in the hippocampus (0.72-fold; Supplemental Figure 1D).

Elevated GAP-43 expression in hnRNP-Q1–deficient cortical neurons increased neurite length and number

The role of GAP-43 in promoting axon growth has been extensively studied (Aigner et al., 1995; Donnelly et al., 2011, 2013; Leu et al., 2010). Therefore we next determined whether elevated GAP-43 protein levels due to hnRNP-Q1 knockdown affect neurite length expression profile in all experimental conditions. The threshold between low and high protein levels for both hnRNP-Q1 and GAP-43 was set at 12,500 A.U. as depicted by the black lines. Red outline indicates the cell population with low levels of hnRNP-Q1 and high levels of GAP-43 that was assessed for increased neurite length and number in Figure 3 (Q1 siRNA + Scr siRNA cells only). (C) Table showing the correlation between hnRNP-Q1 and GAP-43 protein levels. The percentage of total cells in each category is listed, and the highlighted populations (corresponding to the bar graphs in Figure 3) were analyzed for neurite length and number.
in GAP-43 protein levels was directly attributed to loss of hnRNP-Q1 (Figure 2C). The phenotype of high GAP-43 protein levels in hnRNP-Q1–depleted cells (27.6%) was abolished by simultaneous knockdown of GAP-43 (1.6%), demonstrating that GAP-43 can be efficiently depleted (Figure 2C). Additionally, knockdown of GAP-43 by itself did not change the negative correlation between hnRNP-Q1 and GAP-43 protein levels, wherein a substantial percentage of cells (32.6%) still showed high hnRNP-Q1 and low GAP-43 protein levels (Figure 2C). Furthermore, a very small percentage of cells had high hnRNP-Q1 and high GAP-43 protein levels (control: 8.1%; GAP-43: 0.6%; hnRNP-Q1: 7.6%; and hnRNP-Q1 & GAP-43: 3.3%).

To determine whether elevated GAP-43 protein levels due to hnRNP-Q1 knockdown affect neurite length and number, we specifically analyzed the cells within the population that exhibited the characteristic expression pattern for each condition (highlighted in Figure 2C; the average hnRNP-Q1 and GAP-43 protein levels of selected cells are displayed in Supplemental Figure 2B). Shortly after being plated (∼6 h), cultured neurons develop lamellipodia that transform into distinct processes after ∼12 h (Dotti et al., 1988). One of these minor processes is specified to become the axon and was called the “nascent axon” in our studies. The total length and number of all neurites was also quantified. Cells with low levels of GAP-43 protein and high levels of hnRNP-Q1 protein were selected in the control conditions (Scr siRNA + Scr siRNA: 35.8% of cells; Scr siRNA + GAP-43 siRNA: 32.6% of cells); cells with low levels of hnRNP-Q1 protein and high levels of GAP-43 protein were selected in the Q1 siRNA + Scr siRNA condition (27.6% of cells; red outline in Figure 2B); and cells with low levels of both proteins were selected in the Q1 siRNA + GAP-43 siRNA condition (78.8% of cells). Neurons with elevated GAP-43 protein levels following hnRNP-Q1 knockdown correlated with an increased length of the nascent axon by 1.44-fold (31.00 to 44.76 μm; Figure 3, A and D), total length of all the neurites by 1.66-fold (86.11 to 143.12 μm; Figure 3, B and E), and number of neurites per cell by 1.44-fold (4.84 to 6.96 neurites, Figure 3, C and F). Additionally, simultaneously knocking down GAP-43 rescued all three phenotypes back to control levels, which demonstrates that increased GAP-43 protein levels are responsible for the neuritic and axonal phenotypes in hnRNP-Q1–deficient neurons (Figure 3, A–F). The nascent axon length, total neurite length, and neurite number of Q1 siRNA + Scr siRNA cells (unpublished data). These results support our model that increased axon growth in hnRNP-Q1–depleted cells is attributed to elevated GAP-43 expression.

FIGURE 3: Increased cortical neuron nascent axon length, total neurite length, and neurite number due to increased GAP-43 protein expression upon hnRNP-Q1 knockdown. (A) Average nascent axon length, (B) total neurite length, and (C) neurite number of selected neurons from Figure 2 were quantified. n = 7, Scr + Scr: 62 out of 173 cells; Scr + GAP-43: 59 out of 181 cells; Q1 + Scr: 47 out of 170 cells; and Q1 + GAP-43: 145 out of 184 cells from seven independent experiments. One-way ANOVA, Tukey’s posthoc, nascent axon length values: Scr + Scr vs. Scr + GAP-43, p = 0.8423; Scr + Scr vs. Q1 + Scr, p = 0.0004; Scr + Scr vs. Q1 + GAP-43, p = 0.5784; Scr + GAP-43 vs. Q1 + Scr, p < 0.0001; Scr + GAP-43 vs. Q1 + GAP-43, p = 0.9921; Q1 + Scr vs. Q1 + GAP-43, p < 0.0001; total neurite length values: Scr + Scr vs. Scr + GAP-43, p = 0.9884; Scr + Scr vs. Q1 + Scr, p < 0.0001; Scr + Scr vs. Q1 + GAP-43, p = 0.2956; Scr + GAP-43 vs. Q1 + Scr, p < 0.0001; Scr + GAP-43 vs. Q1 + GAP-43, p = 0.5334; Q1 + Scr vs. Q1 + GAP-43, p < 0.0001; neurite number values: Scr + Scr vs. Scr + GAP-43, p = 0.9707; Scr + Scr vs. Q1 + Scr, p = 0.0021; Scr + Scr vs. Q1 + GAP-43, p = 0.4028; Scr + GAP-43 vs. Q1 + Scr, p = 0.0098; Scr + GAP-43 vs. Q1 + GAP-43, p = 0.1699; Q1 + Scr vs. Q1 + GAP-43, p < 0.0001. (D–F) Cumulative distribution plots for each measurement of selected neurons from Figure 2.
Elevated GAP-43 expression in hnRNPA1–deficient N2a cells increased process extension

We next determined whether hnRNPA1 knockdown also affects the morphology of N2a cells. N2a cells were transfected with hnRNPA1 #1 or Scr siRNA, GAP-43 or Scr siRNA and Lifeact-GFP and fixed after 72 h. The cells were then processed for immunofluorescence with GAP-43 and hnRNPA1 antibodies, and transfected cells were selected by GFP signal (Figure 4A). Cells were categorized based on their degree of process extension (Supplemental Figure 3A). hnRNPA1 knockdown significantly increased the proportion of cells with processes as compared with control cells (cat. 2 = 21.7%, cat. 3 = 12.3%, cat. 4 = 4.5%, and cat. 2 = 12.7%, cat. 3 = 2.6%, cat. 4 = 0.3%, respectively; Figure 4B). Additionally, simultaneously knocking down GAP-43 partially rescued this phenotype (cat. 2 = 21.1%, cat. 3 = 6.9%; and cat. 4 = 0.8%; Figure 4B), suggesting that repression of GAP-43 expression contributes to hnRNPA1–mediated inhibition of N2a cell process extension and, potentially, differentiation. Interestingly, the cell media needed to be changed frequently to rescue this phenotype, and N2a cells transfected with just hnRNPA1 #1 shRNA demonstrated enhanced process extension as compared with control cells (cat. 2 = 27.71%, cat. 3 = 35.67%, and cat. 4 = 18.79%, and Cat 2 = 28.2%, Cat 3 = 15.7%, and Cat 4 = 3.2%, respectively; Supplemental Figure 3B), suggesting that hnRNPA1 depletion leads to increased secretion of growth factors.

hnRNPA1 directly binds a GQ sequence in the 5′-UTR of the Gap-43 mRNA through the RGG box

To investigate a possible translational mechanism for how hnRNPA1 represses GAP-43 expression, we first determined whether hnRNPA1 interacts with endogenous Gap-43 mRNA. We overexpressed 3×Flag-mCherry-hnRNPA1 in N2a cells, and hnRNPA1 was immunoprecipitated with anti-Flag agarose beads. Copurifying mRNAs were assessed by qRT-PCR and normalized to levels of the non hnRNPA1 target γ-actin mRNA. Gap-43 mRNA was significantly enriched (2.89-fold) in hnRNPA1 pellets as compared with γ-actin mRNA but not Gapdh mRNA (1.07-fold), suggesting that hnRNPA1 forms a complex with Gap-43 mRNA (Figure 5A). Biotin pull-down experiments were performed to test whether hnRNPA1 directly interacts with Gap-43 mRNA and to identify the Gap-43 mRNA sequences that are required for hnRNPA1 binding. Biotinylated probes corresponding to the Gap-43 5′-UTR, coding region and 3′-UTR were in vitro transcribed along with the γ-actin 3′-UTR as a negative control (Figure 5B). Equimolar concentrations of the RNA probes were incubated with recombinant glutathione S-transferase (GST) or GST-hnRNPA1 protein, and the probes were precipitated with NeutraVidin agarose beads. Copurifying protein was assessed by GST immuno blot. GST-hnRNPA1 but not GST was precipitated with probes corresponding to all three regions of the Gap-43 mRNA but not with the γ-actin 3′-UTR probe (Figure 5C), demonstrating that hnRNPA1 directly interacts with Gap-43 mRNA. Interestingly, we observed that the 5′-UTR precipitated the most GST-hnRNPA1, followed by the coding region, and then the 3′-UTR (Figure 5C). Thus we focused our analysis on a predicted GQ sequence in the 5′-UTR region of Gap-43 mRNA, because these structures have previously been demonstrated to repress translation (Bugaout and Balasubramanian, 2012). The predicted GQ sequence that we assessed had the highest G Score of all predicted GQs in mouse Gap-43 mRNA (G Score = 42, QGRS Mapper [Kikin et al., 2006; Supplemental Figure 4A]). Human Gap-43 mRNA also has a predicted GQ with a high G Score in the 5′-UTR, suggesting a conserved mechanism (G Score = 36, QGRS Mapper [Kikin et al., 2006; Supplemental Figure 4B]). We observed that deleting the Gap-43 5′-UTR GQ sequence (5′GGGAGGGAGGGAGGGA-3′) almost completely abolished GST-hnRNPA1 binding to the 5′-UTR (reduced by 88%; Figure 5C). Because hnRNPA1 appears to bind to multiple regions of the Gap-43 mRNA, we next determined whether deleting the 5′GQ affects hnRNPA1 binding to full-length (FL) Gap-43 mRNA. Biotinylated probes corresponding to FL Gap-43 mRNA with and without the 5′GQ were in vitro transcribed (Figure 5B) and used for biotin pull-down experiments. Deleting the 5′GQ reduced GST-hnRNPA1 binding to FL Gap-43 mRNA by 41%, suggesting that this sequence is a major hnRNPA1–binding site (Figure 5D). Additionally, GST-hnRNPA1 binds to the 5′GQ better than to either a 12-nucleotide or 30-nucleotide poly(A) probe (Supplemental Figure 4C). These results suggest that the 5′GQ is a major hnRNPA1–binding site and
Given that RGG box domains have been demonstrated to specifically bind GQ-forming mRNA sequences (Schaeffer et al., 2001; Menon and Mihaielus, 2007; Menon et al., 2008; Stefanovic et al., 2015b), we next determined whether hnRNP-Q1 interacts with the 5′GQ through the RGG box domain. We overexpressed 3×-Flag-mCherry, 3×Flag-mCherry/hnRNP-Q1, 3×Flag-mCherry/hnRNP-Q1 ΔRGG box and 3×Flag-mCherry/hnRNP-Q1 RGG box in N2a cells for ~16 h. Biotin pull-down experiments were then performed with the 5′GQ probe and the N2a cell lysates. Copurifying protein was assessed by Flag immunoblot. FL hnRNP-Q1 (3×Flag-mCherry/hnRNP-Q1) and the RGG box domain (3×Flag-mCherry/hnRNP-Q1 RGG Box) both bound the 5′GQ probe, while hnRNP-Q1 lacking the RGG box domain (3×Flag-mCherry/hnRNP-Q1 deltaRGG box) did not (Supplemental Figure 4D). These results demonstrate that the hnRNP-Q1 RGG box is necessary and sufficient to bind the Gap-43 5′GQ. We also used fluorescence spectroscopy to determine the binding affinity. We designed a fluorescently labeled Gap-43 5′GQ RNA probe in which the adenine at position four was replaced with 2-aminopurine (2AP), which is a highly fluorescent analogue of adenine whose steady-state fluorescence is sensitive to changes in the microenvironment (Serrano-Andres et al., 2006; Bharill et al., 2008). A sample of 150 nM 2AP-labeled Gap-43 5′GQ RNA probe was prepared in 10 mM cacodylic acid buffer (pH 6.5) and 50 nM increments of the hnRNP-Q1 RGG box peptide were titrated while the changes in the steady-state 2AP fluorescence were monitored (Figure 5E). The resulting binding curves were fitted with Eq. 1 (Materials and Methods) to reveal a dissociation constant (K_d) of 131 ± 14 nM for the complex formed between the Gap-43 5′GQ RNA probe and the hnRNP-Q1 RGG box (Figure 5E). These experiments were performed in triplicate, and the reported error represents the SD of the K_d from the three independent measurements.

The Gap-43 5′-UTR GQ sequence folds into a GQ structure

We next determined whether the Gap-43 5′GQ folds into a GQ structure, as predicted by the GQ prediction software GQRS Mapper (Kikin et al., 2006). The Gap-43 5′GQ probe (with a linker, 5′-GGAGGAGGAGGAGGGA+GAGC-3′) was in vitro transcribed, purified by electrophoresis, and run on a denaturing polyacrylamide gel to verify probe purity (Supplemental Figure 5A). We first analyzed 5′GQ probe folding by 20% nondenaturing polyacrylamide gel with various KCl concentrations (0–150 mM). A single band was observed at all KCl concentrations investigated, indicating that a single GQ conformation was adopted (Supplemental Figure 5B). However, there was a small shift in the band position after KCl was added, which supports potassium-driven stability of the GQ structure (Supplemental Figure 5B).

We then used one-dimensional (1D) 1H NMR spectroscopy to analyze imino proton resonances. Imino proton resonances in the 10–12 ppm region have been assigned to guanine imino protons engaged in Hoogsteen base pairs within individual G-quartets and are considered signatures of GQ structure formation (Furtig et al., 2003; Menon et al., 2008; Namibi et al., 2011). While DNA GQs require the presence of potassium ions for folding, RNA GQs of identical sequence can fold in the absence of these ions but have lower stability (Joachimi et al., 2009). Resonances are present in the 10–12 ppm region even in the absence of KCl, indicating GQ formation within the 5′GQ probe (Figure 6A). The intensity of these resonances increased upon the addition of KCl, demonstrating that the structure is stabilized by K⁺ ions (Figure 6A). A mutant Gap-43 5′GQ probe (with a linker, 5′-GGAGGAGGAGGAGGGA+GAGC-3′) was
also synthesized, in which guanine nucleotides predicted to be engaged in GQ formation were replaced with cytosine nucleotides, and the secondary structure was analyzed by 1D $^1$H NMR spectroscopy. As expected, the GQ imino proton resonances are no longer present in the 10–12 ppm region even in the absence of KCl. The CD spectra of the Gap-43 5′GQ RNA probe in the presence of increasing KCl concentrations were acquired, and the results fitted the signature parallel GQ curve (negative peak at ∼240 nm and a positive peak at ∼265 nm). (C) UV spectroscopic thermal denaturation of the Gap-43 5′GQ RNA probe. Inset: fit of the main hypochromic transition present in the UV thermal denaturation profile of the Gap-43 5′GQ RNA probe with Eq. 4 (Materials and Methods) from which the following thermodynamic parameters were determined: $\Delta H^o = -64.3 \pm 0.1$ kcal/mol, $\Delta S^o = -183.2 \pm 0.1$ cal/mol K and $\Delta G^o = -9.6 \pm 0.1$ kcal/mol. (D) Gap-43 5′GQ RNA probe melting temperature at 5 mM KCl as a function of the RNA concentration. (E) Arrangement of the predicted GQ structure within the Gap-43 5′GQ RNA probe. QGRS Mapper software was used for prediction (Kikin et al., 2006).

FIGURE 6: The Gap-43 5′-UTR GQ sequence folds into a stable, parallel, intramolecular GQ Structure. (A) 1D $^1$H NMR spectroscopy with the Gap-43 5′GQ RNA probe revealed that imino proton resonances are present in the 10–12 ppm region even in the absence of KCl. (B) CD spectra of the Gap-43 5′GQ RNA probe in the presence of increasing KCl concentrations were acquired, and the results fitted the signature parallel GQ curve (negative peak at ∼240 nm and a positive peak at ∼265 nm). (C) UV spectroscopic thermal denaturation of the Gap-43 5′GQ RNA probe. Inset: fit of the main hypochromic transition present in the UV thermal denaturation profile of the Gap-43 5′GQ RNA probe with Eq. 4 (Materials and Methods) from which the following thermodynamic parameters were determined: $\Delta H^o = -64.3 \pm 0.1$ kcal/mol, $\Delta S^o = -183.2 \pm 0.1$ cal/mol K and $\Delta G^o = -9.6 \pm 0.1$ kcal/mol. (D) Gap-43 5′GQ RNA probe melting temperature at 5 mM KCl as a function of the RNA concentration. (E) Arrangement of the predicted GQ structure within the Gap-43 5′GQ RNA probe. QGRS Mapper software was used for prediction (Kikin et al., 2006).
FIGURE 7: hnRNP-Q1 represses endogenous Gap-43 mRNA translation. (A) N2a cells were transfected with hnRNP-Q1 #1 or Scr siRNA for 72 h, starved of methionine for 1 h, and labeled with the methionine analogue AHA or AHA + anisomycin (Anis) for 2 h. AHA incorporated into newly synthesized proteins was labeled with biotin, endogenous GAP-43 protein was immunoprecipitated, and newly synthesized GAP-43 protein was visualized by immunoblot with streptavidin and anti GAP-43. Top, the streptavidin signal; middle, total GAP-43 and AHA GAP-43/total GAP-43 merged signals; and bottom, α-tubulin signal from 5% input. *, Nonspecific bands. Quantification of (B) AHA GAP-43 protein levels normalized to total α-tubulin protein levels from 1 or 5% input and (C) total AHA protein levels from 1% input normalized to total α-tubulin protein levels from 1 or 5% input. n = 3, two-way ANOVA, Tukey’s posthoc, GAP-43 p values: Scr + AHA vs. Scr + Anis, p < 0.0001; Scr + AHA vs. Q1 + AHA, p < 0.0001; Scr + AHA vs. Q1 + Anis, p < 0.0001; Scr + Anis vs. Q1 + AHA, p < 0.0001; Scr + Anis vs. Q1 + Anis, p = 0.6392; Q1 + AHA vs. Q1 + Anis,
(1.62-fold, quantified from 1% input; Figure 7C), suggesting that hnRNP-Q1 regulates a large subset of mRNA transcripts. hnRNP-Q1 knockdown was quantified in Supplemental Figure 6B.

Proximity ligation assays were also performed to confirm that hnRNP-Q1 represses endogenous Gap-43 mRNA translation (David et al., 2012; tom Dieck et al., 2012). N2a cells were transfected with hnRNQ-Q1 #1 or Scr siRNA and Lifeact-GFP; 72 h later, the cells were pulsed with puromycin for 5 min. Media without puromycin was used as a control. Excess puromycin was extracted, the cells were fixed, and proximity ligation reactions were performed with GAP-43 and puromycin antibodies. α-Tubulin and puromycin antibodies were used as a control. The GAP-43 or α-tubulin and puromycin antibodies were bound by secondary antibodies conjugated to oligonucleotides. If a GAP-43 or α-tubulin oligonucleotide was within 30-40 nM of the puromycin antibody, which occurred when puromycin antibodies were bound by secondary antibodies conjugated to oligonucleotides. If a GAP-43 oligonucleotide was within 30-40 nM of the puromycin antibody, which occurred when puromycin was incorporated into a GAP-43 or α-tubulin peptide chain undergoing translation, the oligonucleotides from each secondary antibody were ligated together to form a closed loop. The oligonucleotide loop was then amplified by rolling circle amplification, and fluorescently labeled oligonucleotides were hybridized to the product. Transfected cells were selected by GFP signal (Figure 7D).

hnRNQ-Q1 knockdown significantly increased the volume and intensity of GAP-43 proximity ligation puncta (5.30- and 5.13-fold, respectively; Figure 7, E and F), and the no-puromycin controls demonstrated reduced signal (volume: Scr: 0.18-fold, Q1: 0.42-fold; intensity: Scr: 0.19-fold, Q1: 0.45-fold; Figure 7, E and F). However, hnRNQ-Q1 knockdown did not affect the volume or intensity of α-tubulin proximity ligation puncta (0.95- and 0.93-fold, respectively; Figure 7, E and F), and the no-puromycin controls demonstrated reduced signal (volume: Scr: 0.19-fold, Q1: 0.22-fold; intensity: Scr: 0.19-fold, Q1: 0.23-fold; Figure 7, E and F). These results further suggest that hnRNQ-Q1 represses Gap-43 mRNA translation but not global translation.

hnRNQ-Q1 represses Gap-43 mRNA translation through the 5′-UTR GQ

AHA pulse-labeling experiments were also performed to determine whether the 5′GQ was involved in hnRNQ-Q1-mediated repression of Gap-43 mRNA translation. To perform these experiments, we first created a construct with the following cassette: Gap-43 5′-UTR with or without 5′GQ)-3×Flag-mCherry-Gap-43 coding region-Gap-43 3′-UTR (FL or 5′GQ reporter). N2a cells were transfected with the FL or 5′GQ reporter constructs ~56 h after hnRNQ-Q1 #1 or Scr siRNA transfection. After ~16 h, the cells were starved of methionine for 1 h and labeled with the methionine analogue AHA for 2 h. AHA incorporated into newly synthesized proteins was labeled with biotin, 3×Flag-mCherry-tagged GAP-43 was immunoprecipitated with anti-Flag agarose beads, and newly synthesized 3×Flag-mCherry-tagged GAP-43 was visualized by immunoblot with streptavidin and anti-Flag (Figure 8A). The results revealed that hnRNQ-Q1 depletion increased the translation of the FL reporter by 1.50-fold (Figure 8B). Additionally, the 5′GQ reporter was less sensitive to hnRNQ-Q1 repression, but hnRNQ-Q1 knockdown did significantly increase 5′GQ reporter translation (Scr: 2.64-fold, Q1: 3.39-fold; Figure 8B). These results suggest that hnRNQ-Q1 represses Gap-43 mRNA translation in a 5′GQ-dependent manner but indicate that additional Gap-43 mRNA sequences, potentially additional GQs, may also contribute to this process. hnRNQ-Q1 knockdown was quantified in Supplemental Figure 7A, and a representative example of construct overexpression is shown in Supplemental Figure 7B. In further support of our interpretation of the AHA pulse-labeling experiments, the 5′GQ reporter was expressed at a higher basal rate in N2a cells than the FL reporter (1.28-fold; Figure 8C).

Luciferase assays were also performed to support the AHA pulse-labeling findings. The Gap-43 5′-UTR with or without the 5′GQ sequence was inserted upstream of the firefly luciferase coding region (5′ or 5′GQ constructs). N2a cells were transfected with hnRNQ-Q1 #1 or Scr siRNA, the 5′ or 5′GQ firefly luciferase reporter constructs, and a Renilla luciferase construct. Luciferase assays were performed after 72 h. The results revealed that hnRNQ-Q1 depletion increased the expression of the 5′ reporter as demonstrated by a 1.57-fold increase in luminescence (Figure 8D). Additionally, the 5′GQ reporter was less sensitive to hnRNQ-Q1 repression, but hnRNQ-Q1 knockdown did significantly increase 5′GQ reporter expression (Scr: 2.61-fold, Q1: 3.05-fold; Figure 8D). These findings support the results from the AHA pulse-labeling experiments, which suggest that the 5′GQ and likely additional Gap-43 mRNA sequences contribute to translation inhibition by hnRNQ-Q1. Additionally, luciferase assays were performed with a construct that had just the 5′GQ inserted upstream of the firefly luciferase coding region. N2a cells were transfected with hnRNQ-Q1 #1 or Scr siRNA, the 5′GQ or empty vector firefly luciferase reporter constructs and a Renilla luciferase construct, and luciferase assays were performed after 72 h. The results demonstrate that inserting only the 5′GQ was sufficient to repress luciferase expression as compared with the empty vector (0.56-fold) and that knocking down hnRNQ-Q1 relieves this repression (0.81-fold, Figure 8E). Additionally, expression

\[ p < 0.0001; \text{total p values: Scr + AHA vs. Scr + Anis, } p = 0.0048; \text{Scr + AHA vs. Q1 + AHA, } p = 0.0188; \text{Scr + AHA vs. Q1 + Anis, } p = 0.0111; \text{Scr + Anis vs. Q1 + AHA, } p < 0.0001; \text{Scr + Anis vs. Q1 + Anis, } p < 0.0001; \text{Q1 + AHA vs. Q1 + Anis, } p = 0.0002. \] 

(D) N2a cells were transfected with hnRNQ-Q1 #1 or Scr siRNA for 72 h and incubated with or without puromycin (Puro) for 5 min. The cells were fixed, and actively translating GAP-43 and α-tubulin were detected by proximity ligation. Representative images are shown. Scale bar: 10 μm. Quantification of (E) proximity ligation puncta volume/total cell volume and (F) proximity ligation puncta signal intensity/total cell volume. n = 3, GAP-43: Scr + Puro, 126 cells; Scr + Puro, 105 cells; Scr– Puro, 107 cells; Q1 + Puro, 109 cells; α-tubulin: Scr + Puro, 107 cells; Q1 + Puro, 109 cells; Scr– Puro, 113 cells; Q1 + Puro, 118 cells from three independent experiments, two-way ANOVA, Tukey’s posthoc, Gap-43 volume p values: Scr + Puro vs. Scr – Puro, p = 0.0419; Scr + Puro vs. Q1 + Puro, p < 0.0001; Scr + Puro vs. Q1 – Puro, p = 0.02397; Scr – Puro vs. Q1 + Puro, p < 0.0001; Scr – Puro vs. Q1 – Puro, p = 0.8760; Q1 + Puro vs. Q1 – Puro, p < 0.0001; 5′-UTR volume p values: Scr + Puro vs. Scr – Puro, p < 0.0001; Scr + Puro vs. Q1 + Puro, p = 0.9408; Scr + Puro vs. Q1 – Puro, p < 0.0001; Scr – Puro vs. Q1 + Puro, p < 0.0001; Scr – Puro vs. Q1 – Puro, p = 0.9826; Q1 + Puro vs. Q1 – Puro, p < 0.0001; Gap-43 intensity p values: Scr + Puro vs. Scr – Puro, p = 0.0318; Scr + Puro vs. Q1 + Puro, p < 0.0001; Scr + Puro vs. Q1 – Puro, p < 0.0001; Scr – Puro vs. Q1 + Puro, p < 0.0001; Scr – Puro vs. Q1 – Puro, p < 0.0001; 5′-GQ intensity p values: Scr + Puro vs. Scr – Puro, p < 0.0001; Scr + Puro vs. Q1 + Puro, p = 0.8904; Scr + Puro vs. Q1 – Puro, p < 0.0001; Scr – Puro vs. Q1 + Puro, p < 0.0001; Scr – Puro vs. Q1 – Puro, p = 0.9753; Q1 + Puro vs. Q1 – Puro, p < 0.0001.

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of the empty vector showed a nonsignificant trend toward being slightly increased upon hnRNP-Q1 knockdown (1.23-fold; Figure 8E) suggesting that the poly(A) tail may contribute to this mechanism (Svitkin et al., 2013), but the effect of the endogenous Gap-43 poly(A) tail should be assessed before any conclusions can be made. Nonetheless, these findings demonstrate that the 5′GQ is involved in hnRNP-Q1–mediated inhibition of Gap-43 mRNA translation.

**DISCUSSION**

In this study, we have identified Gap-43 mRNA as a novel target that is translationally repressed by the mRNA-binding protein hnRNP-Q1. These findings contribute to the growing literature about the role of hnRNP-Q1 in regulating translation. hnRNP-Q1 has previously been demonstrated to repress cap-dependent translation of RhoA and YB-1 mRNAs (Xing et al., 2012; Lyabin et al., 2013). However, the specific mechanism of hnRNP-Q1 binding and translation regulation has yet to be uncovered. Here we further identified a predicted GQ sequence in the 5′-UTR of Gap-43 mRNA and determined that it folds into a stable, parallel, intramolecular GQ structure. Additionally, this sequence is involved in hnRNP-Q1–mediated translation repression of Gap-43 mRNA as demonstrated by AHA pulse labeling and luciferase assays. Furthermore, hnRNP-Q1 appears to bind the 5′GQ with higher affinity than poly(A) sequences, and luciferase assays reveal that the poly(A) tail represses expression to a lesser extent than the 5′GQ, suggesting a novel mechanism. In support of this, we have previously demonstrated that hnRNP-Q1 represses RhoA mRNA translation by binding non-poly(A) sequences (Xing et al., 2012). We have also demonstrated that, as in other GQ mRNA-binding proteins, the hnRNP-Q1 RGG box domain is sufficient for the recognition of the Gap-43 5′GQ. Taken together, these results suggest that hnRNP-Q1 is a novel GQ-binding protein and point to a potential mechanism for hnRNP-Q1–mediated translational regulation. GQs proximal to the 5′ cap have previously been shown to inhibit translation by blocking ribosome assembly or scanning (Bugaut and Balasubramanian, 2012). Therefore hnRNP-Q1 may bind to the 5′GQ of Gap-43 mRNA and prevent ribosome assembly or scanning. hnRNP-Q1 may repress the translation of YB-1 by a similar mechanism. YB-1 mRNA is predicted to contain a GQ with a moderately high G-Score in its 5′-UTR region (G-Score = 20, QGRS Mapper [Kikin et al., 2006]). In contrast, RhoA mRNA

\[ p < 0.0001; \text{Scr + FL vs. Q1 + FL}, p = 0.0127; \text{Scr + FL vs. Q1 + ΔGQ}, p < 0.0001; \text{Scr + ΔGQ vs. Q1 + FL}, p < 0.0001; \text{Scr + ΔGQ vs. Q1 + ΔGQ}, p = 0.0011; \text{Q1 + FL vs. Q1 + ΔGQ}, p < 0.0001. \]  

(C) The 3×Flag-mCherry–tagged GAP-43 reporter constructs were overexpressed in N2a cells for ~16 h, and reporter expression was visualized by Flag immunoblot. n = 5, one-sample t test, p value = 0.0177. (D) N2a cells were transfected with hnRNP-Q1 #1 or Scr siRNA, the 5′ or 5′ΔGQ firefly luciferase construct, and a Renilla luciferase construct for normalization. After 72 h, the cells were transfected with the firefly luciferase construct, and a Renilla luciferase construct for normalization. After 72 h, the cells were transfected with the firefly luciferase construct, and a Renilla luciferase construct for normalization. After 72 h, the cells were transfected with the firefly luciferase construct, and a Renilla luciferase construct for normalization.
translation appears to be regulated in a slightly different manner, because the 3′-UTR is sufficient for hnRNP-Q1–mediated translation repression. However, the 3′-UTR of RhoA mRNA also contains a predicted GQ with a high G- Score (G-Score = 41, QGRS Mapper [Kikin et al., 2006]), and 3′-UTR GQs have also been demonstrated to regulate translation (Arora and Suess, 2011; Stefanovic et al., 2015a). These findings suggest that GQ structures may be the primary interacting motifs for hnRNP-Q1–mediated translational inhibition.

Future studies may address this mechanism in more detail, including identifying the steps needed to repress translation. hnRNP-Q1 may modulate translation through a mechanism similar to FMRP, which interacts with mRNA target GQs when it is phosphorylated at a serine residue near the RGG box domain and is dephosphorylated in order to facilitate translation (Ceman et al., 2003; Muddashetty et al., 2011).

Our results further reveal a novel function for hnRNP-Q1 to control nascent axon and neurite growth in incipient neurons by repressing Gap-43 translation. At 28.5 h, in vitro neurons are beginning to polarize but do not demonstrate the stereotypical axonal Tau enrichment and dendritic MAP2 enrichment. These results suggest that hnRNP-Q1 plays an important role in regulating nascent axon outgrowth by modulating Gap-43 expression. Similar phenotypes have been reported upon hnRNP-Q1 knockdown in primary cortical neurons that have undergone Tau and MAP2 expression polarization (3 and 7 d in vitro) suggesting that hnRNP-Q1 functions to regulate neuron morphology at later stages of neuronal development as well (Chen et al., 2012). Additionally, we demonstrated that hnRNP-Q1–mediated repression of Gap-43 expression inhibits N2a cell process extension. Chen et al. (2012) also reported similar N2a phenotypes upon hnRNP-Q1 knockdown but demonstrated that their N2a and neuron phenotypes were due to reduced Cdc42 and associated-factor mRNA localization. However, Gap-43 has been demonstrated to work upstream of both Cdc42 and RhoA to regulate cell morphology, suggesting that hnRNP-Q1 may affect actin cytoskeleton dynamics through the coordinated posttranscriptional regulation of Gap-43, Cdc42, and RhoA expression (Aarts et al., 1998; Gauthier-Campbell et al., 2004). These findings suggest that hnRNP-Q1 likely regulates neuron morphology and function by modulating multiple mRNA targets. In support of this, Chen et al. (2012) performed a microarray analysis for hnRNP-Q1–interacting mRNAs and determined that hnRNP-Q1 interacts with ~10% of the mRNAs that were interrogated (2250 mRNAs). Additionally, gene ontology analysis reveals that several of the mRNA targets encode proteins involved in neuron and synapse function, protein synthesis, and cytoskeletal regulation (Chen et al., 2012). These results suggest that hnRNP-Q1 interacts with and potentially regulates the mRNA localization and/or translation of a specific subset of mRNA targets involved in neuronal development and function. Additionally, Chen et al. (2012) identified two six-nucleotide hnRNP-Q1 consensus sequences, which expands the list of hnRNP-Q1 cis-regulatory elements. hnRNP-Q1 has previously been demonstrated to bind poly(A) RNA ( Mizutani et al., 2000; Svitkin et al., 2013), and our findings demonstrate that it can bind GQ structures. More research is needed to determine whether these different cis-regulatory elements work cooperatively or allow hnRNP-Q1 to perform different functions. For example, mouse Gap-43 mRNA also contains multiple consensus sequences and two poly(A) stretches that may potentially bind hnRNP-Q1. Also, whether hnRNP-Q1 regulates different mRNA targets during specific stages of neuronal development has yet to be determined.

hnRNP-Q1 has also been identified as a component of transport mRNP granules and is localized to both the axons and growth cones of primary cortical neurons ( Bannai et al., 2004; Kanai et al., 2004; Chen et al., 2012). Additionally, Gap-43 mRNA has been detected in the axons and growth cones of differentiated PC12 cells (Smith et al., 2004). These findings suggest that Gap-43 mRNA may be locally translated within axonal growth cones to synthesize new Gap-43 protein in response to axon guidance signals. In support of this, Gap-43 knockdown reduces axon length in dorsal root ganglia neurons, and this phenotype can only be rescued by Gap-43 that is locally translated in axons (Donnelly et al., 2013). Additionally, the AU-rich HuD-binding element is also necessary and sufficient to localize Gap-43 mRNA to axons, suggesting that HuD and IMP1/ZBP1 work cooperatively to stabilize and localize Gap-43 mRNA (Yoo et al., 2013). Our results identify a new factor that regulates Gap-43 expression and suggest that HuD, IMP1/ZBP1, and hnRNP-Q1 may form a complex with Gap-43 mRNA, enabling the precise control of Gap-43 expression. Gap-43 is also required for netrin-1–induced outgrowth and guidance of neocortical callosal axons ( Shen and Meiri, 2013), suggesting that netrin-1 modulates Gap-43 function and may increase Gap-43 expression. Interestingly, netrin-1 induces local translation of β-actin mRNA in an IMP1/ZBP1-dependent manner (Welslhans and Bassell, 2011), implying that netrin-1 may also play a role in regulating the Gap-43 mRNA, HuD, IMP1/ZBP1, and hnRNP-Q1 complex. Future studies may address the interplay between HuD, IMP1/ZBP1, and hnRNP-Q1; determine whether these factors regulate Gap-43 expression locally in axonal growth cones; and investigate the role of netrin-1 on this complex. Our results reveal a novel mechanism for regulating Gap-43 expression and further support the idea that a complex of proteins functions to spatially and temporally regulate Gap-43 expression, enabling the growth cone to respond to specific cues during development.

**MATERIALS AND METHODS**

**Plasmids and siRNA**

Three siRNAs targeting the 3′-UTR of mouse hnRNP-Q1 mRNA, one targeting the 3′-UTR of mouse Gap-43 mRNA, and two Scr sequences were purchased from Eurofins (Huntsville, AL) and annealed according to the manufacturer's directions. Sequences for each siRNA are as follows (including 3′ UU overhangs), hnRNP-Q1 #1 (sense: 5′-GCAGUUCAGGGUGUACAUU-3′, antisense: 5′-UGAUAACCGUUGAAACUGCUU-3′), hnRNP-Q1 #2 (sense: 5′-ACGUUGUUGACGUGCAUUAU-3′, antisense: 5′-AAAGUCGUGCAUUAACCAGCUU-3′), hnRNP-Q1 #3 (sense: 5′-GUUGAAGUUGAGGGCUAGCUU-3′, antisense: 5′-GUAGCGCCUCAACUUCACCUU-3′), hnRNP-Q1 Scr (sense: 5′-GCGCUUGGAAGCGUGGUAUUU-3′, antisense: 5′-GACUAGCCGCUCAAGCCAUU-3′), Gap-43 (sense: 5′-GCA-GUACUUCGAGGGAAAUUU-3′, antisense: 5′-AAUUUCCCAAGAUGACUGCUU-3′), and Gap-43 Scr (sense: 5′-GUCUAGGCGAACAAUACCUU-3′, antisense: 5′-UCAGACUAUUCUCGAGAUCACUU-3′). shRNA constructs were also generated by inserting the hnRNP-Q1 #1 or Scr siRNA sequences into the pLentilox3.7 vector under the synapsin promoter and yielded a similar degree of knockdown. The 3×Flag-mCherry-hu hnRNP-Q1 construct was described previously (Xing et al., 2012).

**Cell culture and transfection**

N2a cells (American Type Culture Collection, Manassas, VA) were grown in GMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Fisher Scientific) at 5% CO2 and 37°C. N2a cells for immunofluorescence experiments were plated on coverslips coated with 1 mg/ml poly-l-lysine (Sigma Aldrich) in borate buffer (40 mM
boric acid, 10 mM sodium tetraborate, pH 8.5) for 2 h; this was followed by vigorous washing with sterile H2O. N2a cells were transfected with Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. A sample of 100 pmol of siRNA was transfected into cells plated in a 6-well dish and lysed 72 h later for immunoblotting. A sample of 40 pmol of each siRNA and 500 ng Lifeact-GFP or 800 ng shRNA were transfected into cells plated on coverslips in a 12-well dish and fixed 72 h later for immunofluorescence. The medium was changed twice per day for rescue experiments to remove any secreted growth factors.

Timed pregnant C57BL/6J mice were delivered from Charles River, and primary cortical neurons were cultured from the embryos at E16.5. Cortices were dissected from the embryos, trypsinized (0.25%, EDTA-free; Life Technologies) at 37°C, rinsed with warm Hank’s balanced salt solution (HBSS) containing 10 mM HEPES (HBSS/HEPES; Fisher Scientific), and dissociated in MEM (Cellgro/Corning, Manassas, VA) containing FBS (MEM/FBS; Sigma-Aldrich). Primary cortical neurons were transfected with the Amaxa nucleofector II device (Lonza, Allendale, NJ) and the mouse neuron nucleofector kit (Lonza) according to the manufacturer’s protocol. The neurons were washed with warm HBSS/HEPES immediately following the dissection. A sample of 150 pmol of each siRNA and 2.5 μg Lifeact-GFP were transfected into 5 million cells; this was followed by recovery in RPMI (Life Technologies) containing 10% horse serum at 37°C and fixed 28.5 h later for immunofluorescence.

Fluorescence microscopy

Cells were visualized with a 60× Plan-Neofluor objective (Nikon, Melville, NY) on a Nikon Eclipse inverted microscope. Images were acquired with a cooled CCD camera (Photometrics, Tucson, AZ) and Nikon Elements software. Exposure times were kept constant and below saturation for quantitative analysis. Images were deconvolved using AutoQuant X (Media Cybernetics, Bethesda, MD). GAP-43 and hnRNP-Q1 signal intensities in the cell body and longest neurite of cortical neurons were quantified by thresholding the volume of either cell area with the GFP signal and calculating the mean gray area with ImageJ. The mean gray areas of three in-focus stacks were averaged. Neurites were traced, and neurite number and length were quantified using the NeuroJ plug-in for ImageJ. Neurites were defined as any protrusion longer than 6.4 μm, and the longest neurite was called the nascent axon. Immunofluorescence images were prepared by creating easy three-dimensional images with constant lookup table values across all conditions in Imaris (Bitplane, Concord, MA). Heat maps were prepared in ImageJ by setting the lookup table of 8-bit images to fire.

qRT-PCR experiments

RNA was extracted from hnRNP-Q1– or Scr siRNA–transfected lysisates with Trizol (Ambion/Life Technologies), and total mRNAs were reverse transcribed into cDNA with superscriptII reverse transcriptase (Life Technologies) and oligo(dT) primers (Life Technologies) according to the manufacturer’s instructions. Real-time PCR was performed with a LightCycler real-time PCR system and LightCycler SYBR Green I reagent (Roche). Primer sequences were as follows: Gap-43, 5′-ACAGATGTTGCTAAGCC-3′ and 5′-CATTCTGATCTAGCAGGCA-3′; HuD, 5′-AGATGGTGCCTGACTACCCGA-3′ and 5′-CCTTGAGGACGGGGATTT-3′; hnRNP-Q1, 5′-TGAAGCCGCGTTATCACAGG-3′ and 5′-CTGGTTACACGTGACGCA-3′; β-actin, 5′-TGTATCCAACTGGGAGCACA-3′ and 5′-GGGGTTGTAAGGCTCTCAA-3′. Relative quantification of each mRNA was determined based on the standard curve generated with the company and delivered at a concentration of 0.076 mg/ml. The hnRNP-Q1 antibody was tested by immunoblotting at 1:300, similar to Xing et al. (2012) and used for immunofluorescence at 1:100. Immunofluorescence was performed following a standard protocol. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in 1× PBS for 10 min, washed with 1× PBS, permeabilized with 0.2% Triton X-100 in 1× PBS, and washed with Tris-glycine buffer (200 mM Tris-HCl, pH 7.5, 100 mM glycine). Cells were blocked for 1 h, incubated with primary antibody for 1 h, and incubated with secondary antibody for 30 min in 5% BSA in 1× PBS with 0.1% Tween 20 at room temperature. Coverslips were mounted with ProLong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (Life Technologies).

Antibodies, immunoblotting, and immunofluorescence

The following antibodies were used for immunoblotting: hnRNP-Q (R: 1:1000; Sigma-Aldrich), GAP-43 (1:5000; Abcam, Cambridge, MA), γ-actin (1:10,000; Santa Cruz, Dallas, TX), α-tubulin (1:50,000; Sigma-Aldrich), IRDye 680LT donkey anti-mouse (1:20,000; Li-Cor, Lincoln, NE), IRDye 800CW donkey anti-mouse (1:20,000; Li-Cor), and IRDye 800CW donkey anti-rabbit (1:20,000, Li-Cor). Immunoblotting was performed following a standard protocol. Lysates were collected in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% deoxycholate and 0.1% SDS) supplemented with 1× protease inhibitor (Roche, Indianapolis, IN) and 1× RNase inhibitor (Ambion/Life Technologies) unless otherwise noted, Bradford assays were performed, and equal amounts of protein were run on SDS–PAGE gels. Nitrocellulose membranes were blocked with 5% fraction V bovine serum albumin (BSA; Roche) in 1× phosphate-buffered saline (PBS), and primary and secondary antibody incubations were performed in 5% BSA in 1× PBS with 0.1% Tween 20 at room temperature for 2 and 1 h, respectively. An Odyssey infrared imager and IRDye 800CW donkey anti-mouse (1:20,000; Li-Cor), IRDye 680LT donkey anti-mouse (1:20,000; Li-Cor), γ-actin, α-tubulin, and hnRNP-Q1 signal intensities in the cell body and longest neurite were averaged. Neurites were traced, and neurite number and length were quantified using the NeuroJ plug-in for ImageJ. Neurites were defined as any protrusion longer than 6.4 μm, and the longest neurite was called the nascent axon. Immunofluorescence images were prepared by creating easy three-dimensional images with constant lookup table values across all conditions in Imaris (Bitplane, Concord, MA). Heat maps were prepared in ImageJ by setting the lookup table of 8-bit images to fire.
corresponding primers, and all relative concentrations were normalized to β-actin mRNA levels as an internal control.

The 3x-Flag-mCherry or 3x-Flag-mCherry-hu hnRNP-Q1 (Xing et al., 2012) constructs were overexpressed in N2a cells for ~16 h, and Flag-tagged proteins were immunoprecipitated with anti-Flag agarose beads (Sigma-Aldrich). After extensive washing, the pellets were split: one-third for immunoblotting and two-thirds for qRT-PCR. Immunoblots were performed with anti-Flag antibody (1:5000; Sigma-Aldrich) to verify overexpression and immunoprecipitation. Copurifying mRNAs were assessed by qRT-PCR, which was performed as above but also included Gapdh primers (5′-GAGTCT-TACGTGTCTTCAC-3′ and 5′-CCACAATGCAAAATTGTGCAT-3′). mRNA levels with 3x-Flag-mCherry immunoprecipitation were inadequate to quantify, mRNA levels with 3x-Flag-mCherry-hu hnRNP-Q1 immunoprecipitation were first normalized to the input and then to γ-actin mRNA levels.

**Biotin pull-down assays**

Gap-43 and γ-actin sequences were amplified from mouse brain cDNA; and the Gap-43 5′-UTR, coding region, and 3′-UTR were pieced together by overlap extension PCR. The Gap-43 5′-UTR GQ (5′GQ) sequence was deleted by ordering a forward primer lacking the flanking sequences for the amplification primers are as follows: RGG box, 5′-GGGGAGGGAGGGAGGGA+GAGC-3′; and RGG box, 5′-GGGGAGGGAGGGAGGGA+GAGC-3′. The 350 μM RNA samples were prepared in 10 mM cacodylic acid buffer (pH 6.5) in a 90% H2O/10% D2O ratio, and KCl was added to 15 μM of the GQ RNA probe (50 nM) into a fixed concentration of 2AP Gap-43 5′GQ RNA (150 nM), and quenching of the fluorescence signal was recorded as a result of the RGG peptide interacting with the RNA probe (each point was corrected for fluorescence contributions originating from the peptide). A concentration of 1 μM of a synthetic peptide derived from the hepatitis C virus core protein was added to the RNA sample before hnRNP-Q1 RGG box peptide titration to prevent nonspecific binding. The binding dissociation constant (Kd) was determined by fitting the binding curves to the equation

\[
F = 1 + \left( \frac{I_b}{I_s} - 1 \right) \left( \frac{[R]+[P]+[RNA]}{2[RNA]} \right) - \sqrt{\left( \frac{[R]+[P]+[RNA]}{2[RNA]} \right)^2 - 4[PN][RNA]}
\]

where \(I_b/I_s\) represents the ratio of the steady-state fluorescence intensity of the bound and free mRNA, [RNA], is the total concentration of mRNA, and [PN] is the total peptide concentration.

**GQ folding assays**

The Gap-43 5′GQ RNA probe (5′-GGGAGGAGGGAGGGA+G AGC-3′) and the mutated Gap-43 5′GQ RNA probe (5′-GGAGGGAGGGA+GAGC-3′) were in vitro transcribed by T7 RNA polymerase–driven transcription of synthetic DNA templates (TriLink BioTechnologies, San Diego, CA). The RNA probes were purified by 20% polyacrylamide, 8 M urea gel electrophoresis and electroelution. Subsequently, the probes were dialyzed against 10 mM cacodylic acid (pH 6.5). The Gap-43 5′GQ RNA probe and its mutated version were run on a denaturing polyacrylamide gel with a previously purified Pds-95 RNA probe (15 nucleotides) to evaluate the purity.

KCl was added to 15 μM of the Gap-43 5′GQ RNA probe in the range 0–150 mM. The samples were annealed by being boiled for 5 min; this was followed by incubation at room temperature for 10 min. Twenty percent native gels in 0.5× Tris/borate/EDTA buffer were run at 100 V for 3 h at 4°C. Probe conformations were visualized by UV shadowing at 254 nm using an Alphalmager (Alpha Innotech, San Leandro, CA).

**Fluorescence spectroscopy experiments**

Steady-state fluorescence spectroscopy experiments with the 2AP Gap-43 5′GQ RNA probe (5′-GGG2APGGAGGGAGGGA+G AGC-3′) were performed on a Horiba Scientific Fluoromax-4 and accompanying software fitted with a 150-W ozone-free xenon arc lamp. Experiments were performed in a 150 μl sample volume, 3-mm path-length quartz cuvette (Starna Cells, Atascadero, CA). The excitation wavelength was set to 310 nm, the emission spectrum was recorded in the range of 330–450 nm, and the band-pass filters for excitation and emission monochromators were both set to 5 nm. Recombinant hnRNP-Q1 RGG box peptide was synthesized by inserting residues 406–561 into pGEX-2T (GE Healthcare Biosciences), inducing protein synthesis in Rosetta2(DE3)pLysS bacteria (Novagen, Madison, WI), purifying the protein by glutathione affinity, and cleaving off the GST tag with PreScission Protease (GE Healthcare Biosciences). hnRNP-Q1 RGG box peptide was titrated (50 nM) into a fixed concentration of 2AP Gap-43 5′GQ RNA (150 nM), and quenching of the fluorescence signal was recorded as a result of the RGG peptide interacting with the RNA probe (each point was corrected for fluorescence contributions originating from the peptide). A concentration of 1 μM of a synthetic peptide derived from the hepatitis C virus core protein was added to the RNA sample before hnRNP-Q1 RGG box peptide titration to prevent nonspecific binding. The binding dissociation constant (Kd) was determined by fitting the binding curves to the equation

\[
F = 1 + \left( \frac{I_b}{I_s} - 1 \right) \left( \frac{[R]+[P]+[RNA]}{2[RNA]} \right) - \sqrt{\left( \frac{[R]+[P]+[RNA]}{2[RNA]} \right)^2 - 4[PN][RNA]}
\]

where \(I_b/I_s\) represents the ratio of the steady-state fluorescence intensity of the bound and free mRNA, [RNA], is the total concentration of mRNA, and [PN] is the total peptide concentration.

KCl was added to 15 μM of the Gap-43 5′GQ RNA probe in the range 0–150 mM. The samples were annealed by being boiled for 5 min; this was followed by incubation at room temperature for 10 min. Twenty percent native gels in 0.5× Tris/borate/EDTA buffer were run at 100 V for 3 h at 4°C. Probe conformations were visualized by UV shadowing at 254 nm using an Alphalmager (Alpha Innotech, San Leandro, CA).

**GO formation in the Gap-43 5′GQ RNA probe was monitored by 1D 1H NMR spectroscopy at 25°C on a 500-MHz Bruker AVANCE spectrometer. The 350 μM RNA samples were prepared in 10 mM cacodylic acid buffer (pH 6.5) in a 90% H2O/10% D2O ratio, and KCl was titrated in the range 0–150 mM. The water suppression was accomplished using the Watergate pulse sequence (Piotto et al., 1992). Similar experiments were performed for the mutated Gap-43 5′GQ RNA probe in the presence and absence of 150 mM KCl to demonstrate that the structure no longer formed when guanine nucleotides predicted to be engaged in GQ formation were mutated.**

CD spectra were acquired on a Jasco J-810 spectropolarimeter at 25°C, using a 1-mm path-length quartz cuvette (Starna Cells). A
sample of 10 μM Gap-43 5′GQ RNA probe in 10 mM cacodylic acid buffer (pH 6.5) was prepared in a volume of 200 μl. GQ formation was monitored between 200 and 350 nm by titrating KCl in the range 5–150 mM and averaging a series of seven scans with a 1-s response time and a 2-nm bandwidth. The spectra were corrected by subtracting the contributions of the cacodylic acid buffer.

UV spectroscopy thermal denaturation experiments were performed on a Cary 3E UV–VIS Spectrophotometer (Varian, Palo Alto, CA) equipped with a peltier cell. Samples of 200 μl containing variable Gap-43 5′GQ RNA probe concentrations in 10 mM cacodylic acid buffer (pH 6.5) and in the presence of 5 mM KCl were annealed as described above and thermally denatured by varying the temperature in the range 20–95°C, at a rate of 0.2°C/min and monitoring the absorbance changes at 295 nm, wavelength sensitive to GQ denaturation (Mergny et al., 1998). A layer of mineral oil was added to the cuvettes to prevent sample evaporation.

To study whether an intermolecular or intramolecular GQ is formed within Gap-43 5′GQ RNA probe, we performed UV spectroscopy thermal denaturation experiments at variable RNA concentrations ranging from 5 to 50 μM and a fixed KCl concentration (5 mM) and in the presence of 5 mM KCl were annealed at variable RNA concentrations ranging from 5 to 50 μM and a fixed KCl concentration. UV spectroscopy thermal denaturation experiments at variable RNA concentrations ranging from 5 to 50 μM and a fixed KCl concentration were performed as above except for the following. Samples of 2 μg of the 3′xFlag-merry-GAP-43 constructs were transfected –56 h after siRNA transfection and –16 h before AHA labeling, and 100 μg protein from each condition was used in the Click-iT reaction. Anti-Flag-agarose beads (Sigma-Aldrich) were resuspended in IP buffer and added to each condition, and the tubes were rotated at 4°C for 2 h. Newy translated proteins and total GAP-43 protein were detected with IRDye 680LT streptavidin (1:1000; Li-Cor), anti-GAP-43 (1:5000; Sigma-Aldrich), and IRDye 800CW donkey anti-mouse (1:20,000; Li-Cor). α-Tubulin (1:5000; Sigma-Aldrich) was also detected as a loading control.

Proximity ligation

Cells were transfected with 40 pmol hnRNQ-P1 #1 or Scr siRNA 72 h before labeling. The cells were washed with warm DMEM and incubated with DMEM with or without 91 μM puromycin (Sigma-Aldrich) for 5 min at 37°C. Excess puromycin was removed by incubating with cold extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM KCl, 0.015% digitonin) for 20 min followed by wash buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM KCl) for 2 min. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in 1× PBS for 10 min, washed with 1× PBS, permeabilized with 0.2% Triton X-100 in 1× PBS, and washed with Tris-glycine buffer. Cells were blocked in 5% BSA for 1 h at room temperature and incubated with primary antibodies in 5% BSA for 1 h at room temperature (1:1000 GAP-43 [Abcam] + 1:200 puromycin [Developmental Studies Hybridoma Bank, Iowa City, IA] or 1:6000 α-tubulin [Abcam] + 1:300 puromycin [Developmental Studies Hybridoma Bank]). Cells were then washed with 1× PBS, and Duolink proximity ligations were performed according to the manufacturer’s protocol (Sigma-Aldrich). Images were analyzed with Imaris (Bitplane). Cell volume was measured by creating a contour surface, and puncta were selected, and their volume and intensity were measured by setting a threshold.

Luciferase assays

The mouse Gap-43 5′-UTR with and without the 5′GQ sequence or the just 5′GQ sequence by annealing primers, forward: 5′-AGCTTT CACTTTGGGAGGGGAGGGAGGATCCTTTC-3′ and reverse: 5′-CATGGAAGATTGCCTCCCTCCCTCCAAAGATTGAA-3′) were subcloned into pGL3 (Promega) upstream of the firefly luciferase coding region with the HindIII and Ncol sites. Cells were transfected with 40 pmol hnRNQ-P1 #1 or Scr siRNA, 500 ng firefly luciferase construct, and 25 ng Renilla luciferase construct (pRL-CMV; Promega). After 72 h, cells were trypsinized and resuspended in fresh DMEM. A measure of 50 μl of cell lysate in triplicate was processed for luciferase activity with the Dual-Glo luciferase assay.
system (Promega) according to the manufacturer’s protocol and a Veritas microplate luminometer (Turner BioSystems/Promega). Renilla luminescence was used as an internal control.

Normalzation and statistics
hnRNP-Q1 and GAP-43 knockdown immunoblot data were normalized to the internal control α-tubulin and graphed relative to the control condition. hnRNP-Q1 knockdown qRT-PCR data were run in duplicate, and average values were graphed relative to the control condition. Hippocampal qRT-PCR data were normalized to the internal control β-actin and graphed relative to the control conditions. hnRNP-Q1 knockdown immunofluorescence and neurite length and number data were normalized to the average control value. The 3xFlag-mCherry-hu hnRNP-Q1 immunoprecipitation qRT-PCR data were first normalized to the input and graphed relative to γ-actin mRNA levels. AHA pulse-labeling data were normalized to the internal control α-tubulin and graphed relative to the control condition. Proximity ligation data were normalized to the average control value. Firefly luminescence values were normalized to the internal control Renilla luminescence and graphed relative to the control condition. Bar graphs represent the mean ± the SEM. Statistical test and p values for each experiment are given in the figure legends.

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