Quaking Regulates \textit{Hnrnapa1} Expression through Its 3’ UTR in Oligodendrocyte Precursor Cells

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Abstract

In mice, Quaking (Qk) is required for myelin formation; in humans, it has been associated with psychiatric disease. QK regulates the stability, subcellular localization, and alternative splicing of several myelin-related transcripts, yet little is known about how QK governs these activities. Here, we show that QK enhances \textit{Hnrnapa1} mRNA stability by binding a conserved 3’ UTR sequence with high affinity and specificity. A single nucleotide mutation in the binding site eliminates QK-dependent regulation, as does reduction of QK by RNAi. Analysis of exon expression across the transcriptome reveals that QK and hnRNP A1 regulate an overlapping subset of transcripts. Thus, a simple interpretation is that QK regulates a large set of oligodendrocyte precursor genes indirectly by increasing the intracellular concentration of hnRNP A1. Together, the data show that hnRNP A1 is an important QK target that contributes to its control of myelin gene expression.

Introduction

Myelin is a lipid-rich structure that facilitates the propagation of electrical impulses along neuronal axons and protects them from degeneration [1–3]. In the central nervous system (CNS), myelin is produced by oligodendrocytes, glial cells that ensheathe nearby axons with specialized cytoplasmic processes. Defects in the formation and maintenance of myelin cause several devastating diseases in humans, including multiple sclerosis, leukodystrophy, and Pelizaeus-Merzbacher disease.

In the mouse, myelin formation requires QK, a STAR domain RNA-binding protein that regulates gene expression after transcription [4]. The \textit{quaking viable} (\textit{Qk}) mutation, which causes a pronounced tremor, was discovered over forty years ago [3]. The phenotype is caused by the failure to form compact myelin in the CNS and derives from a large deletion lying upstream of the \textit{Quaking} (\textit{Qk}) locus [6]. The \textit{Qk}\textsuperscript{ag} mutation eliminates oligodendrocyte expression of two cytoplasmic QK splice isoforms, QK-6 and QK-7, and reduces expression of a third nuclear isoform, QK-5, primarily in the more severely affected rostral areas [7]. In \textit{Qk}\textsuperscript{ag} mice, expression of QK-6 under the control of the oligodendrocyte-specific proteolipid protein gene (\textit{Plp1}) promoter rescues the myelin defect, demonstrating that QK-6 alone suffices to restore QK function [8]. Other alleles of \textit{Qk} are embryonic lethal, except for the \textit{Qk}\textsuperscript{ag} allele identified by Justice and coworkers that also causes severe demyelination [9].

QK is a multifunctional protein proposed to control myelin formation through three different mechanisms. First, QK stabilizes the myelin basic protein (\textit{Mbp}), microtubule associated protein 1B (\textit{Map1b}), and \textit{p250gpl} mRNAs in the brain or in cultured oligodendrocytes [10–12]. Second, QK controls the subcellular distribution of \textit{Mbp} mRNA, such that an increase in the ratio of nuclear to cytoplasmic QK isoforms drives nuclear retention of \textit{Mbp} mRNA and reduces \textit{Mbp} translation [13]. Finally, QK regulates alternative splicing of \textit{Mbp}, \textit{Plp1}, and myelin associated glycoprotein (\textit{Mag}) mRNAs [14]. \textit{Qk}\textsuperscript{ag} mutant brains display altered isoform ratios of \textit{Mbp}, \textit{Plp1}, and \textit{Mag} transcripts, and splicing of a \textit{Mag} mini-gene is altered in the presence of exogenous QK-5 in cultured COS-7 cells [14]. How QK controls such diverse processes is unclear.

Quaking has recently been implicated in several human psychiatric diseases. Human Quaking (\textit{QKI}) has been proposed to be a candidate gene for schizophrenia (SCZ). Genetic linkage analysis has identified a 0.5 Mb haplotype in the region of \textit{QKI} that segregates with SCZ in a large Swedish pedigree [15]. Moreover, SCZ brains have a number of similarities with the brains of \textit{Qk}\textsuperscript{ag} mice, including downregulation of \textit{QKI} and a number of other myelin-related genes, including \textit{Mbp}, \textit{Mag}, and \textit{PLP1} [16–18]. Additionally, like \textit{Qk}\textsuperscript{ag} brains, SCZ brains contain a number of mRNAs that are present at abnormal splice isoform ratios [19–22]. Some of these, including \textit{Mag}, are also mis-spliced in the \textit{Qk}\textsuperscript{ag} mouse, suggesting that splicing alterations in the \textit{Qk}\textsuperscript{ag} mouse and in SCZ brains could share a common mechanism [16]. Other clinical phenotypes in which disruptions in QK function are thought to contribute to disease include major depressive disorder and \textit{6q} terminal deletion syndrome [23,24].

Despite the importance of QK for myelination and its implications for human health, relatively few direct targets have been identified. Thermodynamic binding analysis and SELEX have shown that the sequence element ACUAAY, alternatively termed the Quaking Star Binding Element (QSBE) or Quaking Response Element (QRE), is required for a high affinity...
**Author Summary**

Myelin is a lipid-rich structure that insulates neuronal axons, facilitating electrical conductance and protecting neurons from degeneration. Myelin comprises multiple compact layers of phospholipid bilayer and specific myelin proteins that occupy distinct positions within the structure. In the central nervous system, an RNA–binding protein termed Quaking is required for formation of compact myelin. Quaking regulates the production of several myelin-related proteins by binding to their mRNAs. Quaking controls the overall levels of these proteins and controls the relative amount of sequence variants of the proteins generated through alternative splicing. Here, we identify a new Quaking mRNA target, the Hnrnpa1 transcript. We show that Quaking regulates the overall level of hnrnpA1. Because hnrnpA1 is itself an RNA regulatory factor and has been implicated in the control of alternative splicing, regulation of hnrnpA1 by Quaking may have consequences for the expression of multiple additional targets. We show that hnRNP A1 and Quaking regulate an overlapping set of transcripts and exons in myelin-forming cells of the central nervous system.

Interaction between purified Quk protein and its target RNA [25,26]. Additionally, a recent cross-link immunoprecipitation study using chemically modified nucleotides detected binding to the sequence ACUAAY and an additional sequence variant AUUAAY [27]. It remains unclear how binding affinity in vitro correlates to binding site occupancy and regulation in vivo. The relative simplicity of the binding determinant, which is predicted to occur approximately once per kb of random RNA sequence, suggests that the binding element may not be sufficient to specify mRNA targets [28]. A single QSBE is present within the 3′ UTR of Mbp mRNA in a region required for post-transcriptional regulation of Mbp expression [25,26]. However, a region of Mag pre-mRNA that has been shown experimentally to drive Quk-dependent splicing events does not contain a QSBE or the variant AUUAAY [14]. Here, we set out to determine whether the Quk binding element mediates Quk-dependent regulation in Mbp and other regulatory targets. We show that Quk regulation of Mbp expression requires the QSBE. Additionally, we identify a novel target, Hnrnpa1 mRNA, and show that Quk regulates Hnrnpa1 expression in a similar fashion to Mbp. Finally, we show that hnRNP A1 contributes to Quk control of myelin gene expression in genome-wide analyses.

**Results**

The QSBE is a functional regulatory element within the Mbp 3′ UTR

It has previously been shown that Quk interacts with a region of the Mbp 3′ UTR and that the core element required for the interaction is the sequence ACUAAY [25,26]. SELEX experiments suggest that a nearby YAAY may also contribute to target selection [26]. We asked whether the QSBE is required for post-transcriptional regulation of mRNA by Quk.

To test whether the QSBE is a required element within the Mbp 3′ UTR, we established a quantitative two-color fluorescence reporter assay in CG4 oligodendrocyte precursor cells, an immortalized cell line (Figure 1A, Figure S1A, S1B, S1C) [29]. CG4 cells can be maintained in culture as progenitors or induced to differentiate into mature oligodendrocytes, can be transfected, and express Quk and a relevant Quk target population. Additionally, their use avoids the potential complications of the Qk6 mouse, in which the genetic lesion lies upstream of the Qk locus and deletes parts of two additional genes [6,30]. A complete Qk knockout is embryonic lethal [31].

We compared expression of a GFP reporter bearing the Mbp 3′ UTR to a reporter bearing a mutant Mbp 3′ UTR harboring a U to G point mutation that blocks binding of Quk to the RNA (Figure 1B) [25]. All other aspects of the constructs were identical. We observed three-fold less reporter mRNA in cells transfected with the mutant construct compared to the wild type (relative expression 0.36±0.07, p = 0.02) (Figure 1C). In contrast, GFP reporter fluorescence increased by a small amount as a result of the mutation (relative expression 1.38±0.20, p = 0.04) (Figure 1C). Dividing the protein expression by the mRNA expression gives a representation of the efficiency of mRNA translation [32]. The QSBE mutant yields four-fold more reporter protein per unit RNA compared to the wild-type reporter (relative value 3.83±0.93, p = 0.03) (Figure 1C). In total, we observed two opposing effects of the QSBE in the Mbp 3′ UTR: a positive effect on mRNA level and a negative effect on the efficiency of translation. These results demonstrate that the QSBE is a functional cis-regulatory element within the Mbp 3′ UTR and are consistent with published findings that Quk positively controls Mbp mRNA stability and negatively controls its translation through nuclear retention of its mRNA [10,13].

Quk interacts with a QSBE within the 3′ UTR of the Hnrnpa1 mRNA

In addition to promoting mRNA stability, Quk regulates alternative splicing. However, the sequence that has been experimentally shown to drive Quk dependent splicing, the Mag splicing control element, does not contain the QSBE sequence or the variant AUUAAY [14]. Moreover, recombinant Quk does not interact with the Mag splicing control sequence or the splicing control sequence of another Quk target, Pph1, by electromobility shift assays [33,34] (Figure S2). The site of Quk binding could lie distal to the minimal Quk-responsive splicing elements that have been described. Alternatively, accessory proteins found in the nucleus might modify Quk binding specificity, bringing it to other targets. A third and simpler possibility is that the effect of Quk on alternative splicing could be a secondary consequence of deregulation of another factor.

To test the final hypothesis, we first determined the ability of Quk to associate with five splicing factor mRNAs (Hnrnpa1, Tra2b, Sf3a5, Ppp4r4b, and Sla7) that contain a QSBE in their 3′ UTRs by RNA-immunoprecipitation from mouse brain lysate. We were unable to detect an interaction between Quk and Sf3a5, Tra2b, Ppp4r4b, and Sla7 mRNAs using an anti-Quk antibody that recognizes all three Quk isoforms (Figure S3). In contrast, we observe association of Quk with Hnrnpa1 mRNA, which contains a single QSBE sequence within a conserved region of its 3′ UTR (Figure 2A), in both uncrosslinked and formaldehyde crosslinked brain lysates (Figure 2B, 2C). In uncrosslinked brain lysates, Quk antibodies immunoprecipitate 20% of the input Hnrnpa1 mRNA, and 3% of control Sf3a1 mRNA, which encodes a core splicingosomal component. The formaldehyde crosslinking experiment controls for the possibility of Quk repartitioning during extract preparation, while the uncrosslinked experiment confirms that the crosslinking agent is not capturing a transient association.

To determine whether Quk binds the QSBE within the Hnrnpa1 3′ UTR with high affinity and specificity, we used gel mobility shift and fluorescence polarization (FP) assays to measure the association of recombinant, purified Quk RNA-binding domain with a 30 nucleotide synthetic RNA derived from the Hnrnpa1 3′ UTR.
UTR and containing the QSBE (Figure 2A). The $K_{d, \text{app}}$ for binding was $36 \pm 6$ nM; a U to G mutation within the QSBE reduced binding by $50$-fold (Figure 2D, 2E). The previously identified Mbp QSBE bound with an apparent $K_d$ of $44 \pm 2$ nM, and the interaction was competed with a 12-nucleotide Hnrnpa1 RNA containing the core ACUAAY motif (Figure 2E).

**QK controls HnRNP A1 mRNA abundance and translational efficiency**

We used the GFP reporter assay to test whether the QSBE from HnRNP A1 functions in cultured CG-4 cells (Figure 3A). The U to G point mutation within the HnRNP A1 3' UTR that eliminated QK binding in vitro (Figure 2D) reduced reporter mRNA level by approximately five-fold (relative expression $0.21 \pm 0.04, p = 0.001$) (Figure 3B). As with the Mbp reporter, the binding site mutation had a small increase on the amount of fluorescent protein produced ($1.36 \pm 0.2, p = 0.001$) (Figure 3C). The same increase was also observed using an independent dual luciferase reporter assay in which the HnRNP A1 3' UTR containing the wild-type or the U-to-G point mutant QSBE was inserted into the Renilla reniformis luciferase 3' UTR ($1.9 \pm 0.5, p = 0.02$) (Figure 3D). Renilla expression also increased in a CG-4 cell line that stably expresses a Qk shRNAmiR ($1.53 \pm 0.05, p = 0.04$) (Figure 3D). We were unable to test the effect of Qk shRNAmiR in the GFP reporter assay because the shRNAmiR construct also expresses GFP.

To obtain a measure of translational efficiency, we divided the normalized reporter fluorescence by the normalized amount of reporter mRNA [35]. Translational efficiency of the QSBE mutant HnRNP A1 3' UTR was increased ($6.58 \pm 1.66, p = 0.014$) compared to the construct without the mutation (Figure 3E), indicating that the QSBE mutation increases the efficiency of translation of the reporter transcript. We were not able to determine reporter mRNA concentration using the luciferase reporter assay because of persistent contaminating reporter DNA. Together the data establish that QK binds the HnRNP A1 3' UTR through the QSBE and that this binding enhances HnRNP A1 mRNA stability but reduces its translation. The results parallel the data collected using the Mbp reporter.

**Figure 1. The QSBE is a regulatory element in the Mbp 3' UTR.** (A) Schematic drawing of the fluorescence reporter assay. (B) Constructs used in reporter assays. The Mbp 3' UTR or the Mbp 3' UTR with the U to G point mutation were inserted into the 3' UTR of phrGFPII-1. tdTomato was unmodified and served as a transfection control. (C) tdTomato and GFP mRNA level were measured by real time RT-PCR from cells co-transfected with the indicated GFP construct and the tdTomato transfection control. The data in the graph are the average and standard deviation of six replicates from two independent experiments. The ratio of green to red fluorescence intensity was determined from nine replicates in three independent experiments. The graph represents the average and standard deviation. The normalized reporter fluorescence was divided by the normalized reporter mRNA to obtain a measure of translational efficiency. Error bars represent the error propagated from the standard deviation of the reporter fluorescence and real time RT-PCR data.

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The reporter constructs isolate 3'-UTR dependent regulation of Hnrnpa1. However, Hnrnpa1 expression is also regulated in 3'-UTR-independent ways. For example, hnRNP A1 is known to regulate its own alternative splicing [36]. Moreover, hnRNP A1 protein stability or indirect regulation of hnRNP A1 transcription would not factor into reporter expression measurements. It is therefore important to determine the overall effect of QK on the amount of endogenous hnRNP A1. We measured Hnrnpa1 mRNA and protein levels in the Qk knockdown line exhibited a four-fold decrease in endogenous Hnrnpa1 mRNA (0.25 ± 0.18, \( p = 0.012 \)), which matches the effect of mutating the QSBE in the Hnrnpa1 3'-UTR reporter. We also observe a four-fold reduction in hnRNP A1 protein (0.24 ± 0.25, \( p = 0.018 \)) in the Qk shRNA\textsuperscript{mkk} line (Figure 4B, 4C), which contrasts with our 3'-UTR reporter data. Thus, we conclude that QK has a net positive effect on hnRNP A1 expression in CG-4 cells, and that QK-dependent regulation of Hnrnpa1 mRNA stability is more important to endogenous hnRNP A1 expression than control of translation efficiency, at least in CG-4 oligodendrocyte precursors.

hnRNP A1 regulates Mag exon 12 alternative splicing

Because hnRNP A1 represses alternative splicing, misregulation of hnRNP A1 is predicted to alter splicing patterns in the Qk\textsuperscript{mkk} mouse brain. Wu et al. [14] identified splicing changes in the Hnrnpa1 3'-UTR region. Also included are the sequences analyzed by gel shift and fluorescence polarization assays, including the U to G point mutant, and the Mbp QSBE for comparison. (A) Mouse, rat, and human Hnrnpa1 3'-UTRs contain the ACUAA element in a conserved UTR region. Also included are the sequences analyzed by gel shift and fluorescence polarization assays, including the U to G point mutant, and the Mbp QSBE for comparison. (B) Hnrnpa1 mRNA co-immunoprecipitated with QK from mouse brain lysate. Lanes are as indicated. (C) Hnrnpa1 mRNA co-precipitated from a lysate prepared from formaldehyde crosslinked mouse brain tissue. Lanes are as indicated. Products were detected in an ethidium bromide stained gel. (D) Electrophoretic mobility shift assays demonstrate a high affinity interaction between QK and the wild type Hnrnpa1 QSBE in vitro. The U to G point mutant, QSBE Mut, did not interact. (E) EMSA and fluorescence anisotropy assays demonstrating an interaction between QK and the Mbp QSBE of similar affinity and competition of the interaction with a 12 nucleotide Hnrnpa1 3'-UTR fragment containing ACUAA. Error bars indicate the standard deviation of five reads of each polarization measurement for each experiment. The error indicated for \( K_{d, app} \) and \( K_{c, app} \) is the standard deviation of at least three experiments.

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Figure 3. QK controls reporter expression through the QSBE in the Hnrrpa1 3’ UTR. (A) Constructs used in reporter assays. The Hnrrpa1 3’ UTR or the Hnrrpa1 3’ UTR with the U to G point mutation was inserted into the 3’ UTR of phrGFPII-1. tdTomato was unmodified and served as a transfection control. For the luciferase assays, the firefly luciferase transfection control was encoded on the same plasmid as the Renilla luciferase. The wild type or mutant Hnrrpa1 3’ UTR was cloned behind Renilla luciferase, and served as a transfection control. For the luciferase assays, the firefly luciferase SV40 poly(A) reporter was inserted into the 3’ UTR and the U-to-G QSBE point mutation. (B) Two color fluorescence reporter assays were assayed as in Figure 1. The RNA data represent the average and standard deviation of five independent replicates. Error bars indicate the standard deviation of five independent replicates for the CG-4 cells or variance of two replicates for the shRNAmiR cell line. Reduction of QK with Qk siRNA caused an increase in the amount of S-Mag compared to L-Mag, resulting in an isoform ratio of 4.7 ± 1.2 (p = 0.04, Figure 5A, 5C). The increase is similar to that previously observed by others in the Qkqk mouse brain, confirming that QK controls Mag splicing in CG-4 cells as it does in brains. CG-4 cells transfected with Hnrrpa1 siRNA displayed an increased isoform ratio of 5.0 ± 1.7 (p = 0.01) (Figure 5A, 5C). The direction of the change is consistent with a role for hnRNP A1 in splicing repression and with our observation that QK has a net positive effect on hnRNP A1 expression in CG-4 cells. Transfection with control, Hnrrpa1, or Sfrs5 siRNAs did not significantly alter the isoform ratio (Figure 5A, Figure S4). The data indicate that hnRNP A1 regulates Mag exon 12 splicing and suggest that QK control of Mag splicing may be indirect. We also examined alternative splicing of Plp1 and Mbp. RT-PCR analysis of Mbp alternative splicing did not reliably confirm the QK-dependence in CG-4 cells (data not shown). To further examine Plp1 splicing in QK knockdown and in hnRNP A1 knockdown CG-4 cells, RT-PCR primers were designed to determine the relative ratio of Plp1, which contains a longer exon 3 variant due to alternative 5’ splice site selection, to Dm20, a transcript containing the shorter exon 3 variant. While QK siRNA reduced the amount of Plp1 compared to Dm20, mirroring the change observed in Qkqk mouse brains, there was no detectable change upon transfection with Hnrrpa1 siRNA (Figure 5B, 5C). The data suggest that for Plp1, QK controls alternative splicing not through hnRNP A1 but, instead, through some other mechanism.

Microarray assay design

Because QK positively regulates hnRNP A1 in CG-4 cells, we anticipated that the isoform ratio of many transcripts in addition to Mag and Plp1 would be altered upon QK knockdown. To test this hypothesis, we used Affymetrix 1.0 ST exon microarrays to assess global changes in gene expression in response to QK depletion (Figure 6A). To reduce the likelihood of detecting off-target effects or changes in gene expression brought about by the clonal selection procedure, we also analyzed cells that were prepared by transient transfection with Qk siRNA (Ambion) along with a GFP-expressing plasmid that allows selection with a different drug than the control siRNA does not (Figure 6B). In parallel, we also analyzed changes caused by depleting hnRNP A1 by transient transfection of a corresponding siRNA. Depletion of hnRNP A1 was confirmed by Western blotting (Figure 6C). Three independent biological replicates were analyzed for each group. In addition to the Qk shRNA, Qk siRNA, and Hnrrpa1 siRNA groups, we also assayed gene expression in control cells treated with non-silencing shRNA, non-silencing siRNA, and untreated CG-4 cells.

Identification of transcripts controlled by QK in oligodendrocyte precursors

We first wished to identify the set of transcripts regulated by QK at the level of transcript abundance. At a False Discovery Rate (FDR) of 0.1 (step-up, p-value 0.0012), 224 transcripts were identified with a p-value at or below the cut-off (Figure 7A, Table
Hierarchical clustering (Pearson’s dissimilarity, centroid method) revealed that gene expression in the three replicates was calculated and the log2 fold change compared to untreated CG-4 cells was determined.

Gene expression changes in the control samples were less correlated, indicating that we have identified changes in gene expression due to the reduction of QK and not to other elements of the experimental protocol. To confirm the array results, we used quantitative RT-PCR to assess gene expression changes in the Qk siRNA stable cell line. Of fourteen transcripts tested, thirteen had a difference in expression by quantitative RT-PCR (Figure 7B). The transcript that had the greatest change in QK knockdown cells was the previously identified QK target Mbp (Figure 7B).

Gene ontology analysis revealed that myelin components were significantly enriched, as predicted from the role of QK in oligodendrocyte precursors. In order to obtain a set of exons predicted to be alternatively spliced upon QK knockdown, we re-analyzed our microarray data using AltAnalyzer [45] and the full Affymetrix probeset annotation. The analysis identified both upregulated and downregulated exons in both data sets. For Qk knockdown CG-4 cells, 78 exons were downregulated and 219 were upregulated, and for Hnpan1 knockdown cells, 93 exons were downregulated 70 were upregulated.

To identify enriched motifs, we determined the frequency of all 5mers contained in the sequences of regulated exons relative to those in Qk siRNA treated cells (correlation coefficient 0.81) (Figure 7C). Qk siRNA and Qk siRNA also clustered closely, as one would predict (correlation coefficient 0.91) (Figure 7C). The correlation between expression changes in the Hnpan1 siRNA and Qk knockdown groups was independent of the False Discovery Rate (Figure S5). The data indicate that there is similarity in the probeset level changes in gene expression between the Qk knockdown and Hnpan1 knockdown samples genome-wide.

We repeated our analysis at the whole transcript level, selecting transcripts regulated by QK (FDR 0.1) and performing the same analysis as for probesets. Again, there is a strong correlation at the whole transcript level between expression changes in the Hnpan1 siRNA and the Qk siRNA samples (Pearson’s correlation coefficient 0.83) (Figure S6). This correlation is of a similar magnitude to the correlation we observe between Qk siRNA and Qk siRNA (Pearson’s correlation coefficient 0.88). These data suggest that QK and hnRNP A1 exert similar pressures on gene expression and alternative splicing on a genome-wide scale in oligodendrocyte precursors.

hnRNP A1 motifs are enriched in QK-responsive exons

hnRNP A1 is a sequence-specific RNA binding protein that regulates the isoform ratio of transcripts that contain a binding site in or near exons that are alternatively spliced [40–42]. It binds to several sequence variants, including the SELEX-derived UAGGG(Δ/U) as well as UAGGU and related sequences [43,44]. Our observation that QK regulates Hnpan1 expression predicts that hnRNP A1 binding sites will be present in the vicinity of QK-responsive exons.

In order to obtain a set of exons predicted to be alternatively spliced upon Hnpan1 or Qk knockdown, we re-analyzed our microarray data using AltAnalyzer [45] and the full Affymetrix probeset annotation. The analysis identified both upregulated and downregulated exons in both data sets. For Qk knockdown CG-4 cells, 78 exons were downregulated and 219 were upregulated, and for Hnpan1 knockdown cells, 93 exons were downregulated 70 were upregulated.

To identify enriched motifs, we determined the frequency of all 5mers contained in the sequences of regulated exons relative to
their frequency in all annotated rat exons (Table S2). To assess statistical significance, we also determined the 5mer frequency from ten sets of an equivalent number of randomly selected exons. Significantly changed 5mers were identified based upon criteria of a greater than 1.5 fold enrichment and a p-value of less than 0.05 when compared to the randomly selected exon set (Table S2). We found a significant enrichment over random of 42 5mers in QK upregulated exons, including the hnRNP A1 motif TAGGT (fold enrichment $FE = 1.54$, $p = 5 \times 10^{-3}$) and the QSBE motif ACUAA ($FE = 1.54$, $p = 8 \times 10^{-7}$, Figure 8A). The A1 motif is similar to an exonic splicing silencer motif identified by Burge and co-workers [46]. There are 149 significant enriched 5mers in QK downregulated exons. These include the hnRNP A1 motif TAGGT ($FE = 1.94$, $p = 3 \times 10^{-3}$), but not the QSBE ACUAA motif.
this motif is enriched relative to all exons, the average of the randomly selected exons also yields a significantly changed p-value. As expected, hnRNP A1 motifs were also enriched in the hnRNP A1 knockdown cells (Figure 8A: upregulated exons; TAGGT, FE = 2.32, p = 4.610^{-2}; downregulated exons: TAGGG, FE = 2.5, p = 1.710^{-5}). The enrichment of the hnRNP A1 motif in QK-responsive exons suggests that many but not all QK-dependent alternative splicing events are mediated indirectly by hnRNP A1. The enrichment of the QK motif is consistent with a direct role for QK in the regulation of some alternative splicing events.

**Discussion**

The RNA binding protein QK controls myelination by governing the stability, subcellular localization, and alternative
Figure 7. Identification of the QK and hnRNP A1 regulatory networks in CG-4 cells. (A) Hierarchical clustering demonstrated high correlation in the gene expression changes between the two knockdown populations. Transcripts altered by QK depletion were identified using a two sample t-test and an FDR of 0.1 (p-value cutoff 0.0012). For the selected transcripts, the expression values of the three biological replicates were averaged and the fold change compared to untransfected CG-4 cells was calculated. The resulting values were clustered (Pearson’s dissimilarity, centroid method), and correlation coefficients were calculated. (B) Real time PCR confirmation of microarray results. RNA was extracted from the Qk shRNAmiR knockdown cell line and from the nonsilencing shRNAmiR control cell line. Real time PCR was performed in triplicate. Expression values were normalized to tubulin as a loading control. Data are the mean of three replicates, expressed at log2 for comparison to the microarray results. (C) QK-dependent probeset expression changes are highly correlated with probeset expression changes caused by hnRNP A1 depletion. Individual probesets with expression changes in response to QK knockdown were identified using a two-sample t-test, comparing the knockdown groups to the control groups. The mean of each group was determined and the difference from the CG-4 cell line was calculated. Values were clustered as in (A). Correlation coefficients were calculated using Partek GS software. Probesets were selected for clustering based solely on their change in the QK knockdown samples.
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splicing of a specific set of myelin-related mRNAs. Relatively few direct QK targets have been characterized, and the mechanism by which QK regulates such diverse processes is unclear. Here, we show that the QSEB sequence—the primary determinant for high affinity QK binding—is required for regulation of Mbp mRNA. Moreover, we show that QK directly regulates the transcript encoding the splicing factor Hsamp1 through a conserved QSEB in its 3’ UTR. The strong correlation between the changes in transcript and proteost abundance upon Qk or Hsamp1 knockdown is consistent with a vertical regulatory relationship and suggests that hnRNP A1 is a primary effector of QK function.

The reporter data reveal that QK has two opposing effects on Mbp and Hsamp1 expression, positively regulating mRNA abundance while negatively regulating translational efficiency. The mode of regulation is consistent with a model where QK stores its targets in a translationally quiescent but stable complex. Such a regulatory scheme would enable a transient burst of translation in response to a signal that releases QK from its targets, such as phosphorylation. Consistent with this hypothesis, Feng and co-workers have shown that QK activity is regulated by tyrosine phosphorylation and that the level of phosphorylation is consistent with a model where QK abundance while negatively regulating translational efficiency.

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The microarray analysis described herein delineates the set of transcripts controlled by QK in the CG-4 oligodendrocyte precursor cell line. The most dramatically changed transcript is the previously characterized QK target Mbp [10,13]. We have also observed striking changes in the expression of several other important myelin genes, suggesting that QK has broad control of transcripts important for myelination. For example, Cnp1 has been implicated in maintaining the integrity of myelinated axons [1]. Fyn stimulates Mbp transcription, suggesting control of Mbp expression by QK is both direct and indirect [48]. Finally, UDP glycosyltransferase 8 (Ugt8) produces myelin sphingolipids, suggesting that QK controls synthesis of the lipid components of myelin as well.

Several labs have demonstrated that hnRNP A1 is a post-transcriptional regulator of gene expression. hnRNP A1 represses alternative splicing when associated with silencing elements near splice sites [40–42]. Additional functions have also been proposed. hnRNP A1 also binds to telomeric DNA, where it is thought to promote telomere elongation [49]. Furthermore, hnRNP A1 is implicated in the Drosha-mediated processing of a primary microRNA [50,51]. It will be interesting to determine whether the effects of QK on gene expression in oligodendrocyte precursors are mediated by hnRNP A1 through any of these mechanisms.

The data also have implications for the mechanism by which hnRNP A1 regulates Mag alternative splicing. An hnRNP A1 motif is present in Mag intron 12 immediately downstream of the 5’-splice site (Figure 8B), suggesting a model in which binding of hnRNP A1 to this motif blocks 5’-splice site recognition, promoting skipping of exon 12. Consistent with this model, our data and the work of others show that reduction of QK or hnRNP A1 increases MAG exon 12 inclusion [14]. Moreover, a point mutation in this hnRNP A1 binding motif has been shown to cause constitutive exon 12 inclusion [14], although we note that this mutation also strengthens the 5’-splice site consensus, which confounds a simple interpretation of this result.

The microarray analysis suggests that some of the effects of QK on splicing may be indirect. In line with these observations, screening the 3’ UTRs of the QK responsive transcripts using the sequence analysis tool Patscan [52] reveals that under a third contain the sequence element required for QK binding (Table S3). Together, these observations suggest numerous indirect changes in gene expression caused by QK depletion. Consistent with this hypothesis, we observe enrichment of 5mer motifs that do not correspond to QK or hnRNP A1 binding motifs in QK-responsive exons (Table S2). These may represent binding sites for other RNA regulators.

Understanding how QK controls its network of transcripts will require a detailed analysis of directly bound QK targets in oligodendrocyte lineage cells. Use of the cross-linked immunoprecipitation technique [53–56] should greatly facilitate such an analysis. A recent study used a variant of the technique, in which chemically modified nucleotides were incorporated into mRNAs in HEK293 cells overexpressing QK to enhance crosslinking [27]. Despite the fact that the mRNAs expressed in HEK293 cells are likely different from those expressed in oligodendrocyte precursors, more than 11–13% overlap when the list of crosslinked transcripts are present the list of crosslinked transcripts, which is substantially different from those expressed in oligodendrocyte lineage cells, 20% of the transcripts identified in our microarray analysis are present the list of crosslinked transcripts, more than the 11–13% overlap when the list of transcripts crosslinked to Pumilio 2 or TNRC6 are analyzed as controls.

Numerous studies have implicated QKI and other myelin genes in SCZ. Thirty-eight genes that have been implicated in SCZ [57] are affected by Qk knockdown, including CNP, MBP, PLP1, and OMG (Table S4). Additionally, SCZ brains have similar splicing defects to those observed in association with QK knockdown, including changes in MAG, NCAM, and EBB3 [16,21,58]. Thus,
the changes in gene expression observed by microarray analysis may provide a link between QKI function and disease.

Gene dosage effects have been implicated in many other human diseases. For example, Pelizaeus-Merzbacher disease is associated with \textit{PLP1} duplication [59]. Intriguingly, copy number variation has been linked to schizophrenia, Charcot-Marie-Tooth disease, and autism, suggesting that small variations in the dosage of important myelin related genes can have significant clinical outcome [60–62].

In summary, we have identified a new direct QK target, the \textit{Humpal} transcript, in oligodendrocyte lineage cells. Additionally, we have identified the set of transcripts directly or indirectly controlled by QK and have shown that hnRNP A1 co-regulates part of this set. The results suggest that the importance of QK for myelin formation lies not only in the identity of major direct targets, but also in the network of secondary targets under QK control. They also demonstrate that QK is a prime regulatory factor controlling gene expression in oligodendrocyte precursors.

Figure 8. hnRNP A1 motifs are enriched in QK-responsive exons. (A) Relative 5mer frequencies in exons that change in response to QK or hnRNP A1 knockdown were binned according to fold enrichment compared to frequency in all exons. The position of hnRNP A1 and QK motifs is annotated by an arrow. The significance of the enrichment was determined by comparison to 5mer fold enrichment in ten groups of an equivalent number of exons selected at random. (B) An hnRNP A1 motif (gray box) is present in \textit{Mag} intron 12 immediately downstream of the 5’-splice site (arrow). The position of the mutation that causes constitutive \textit{Mag} exon 12 inclusion is boxed [14].

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and that hnRNP A1 is a large contributor to its effects on alternative splicing.

Materials and Methods

Ethics statement

Brain tissue was harvested from mice in accordance with protocols approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. The animal care program complies with Federal and State laws and the PHS policy on Humane Care and Use of Laboratory Animals. The experiments were designed in order to minimize the number of animals used.

Cell culture and transfections

The CG-4 rat oligodendrocyte precursor and B104 cell lines were a gift from Lynne Hudson and were cultured according to Louis et al. [29]. CG-4 cells were maintained as undifferentiated progenitors in the presence of 30% B104 conditioned medium for the duration of the assays except as noted otherwise. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to instructions from the manufacturer. Where indicated, 24 hours after transfection, transfected CG-4 cells were selected by incubation with 1500 ng/μl G418 (Geneticin, Invitrogen) for 48 to 72 hours.

To generate stable cell lines, cells were transfected as above with the indicated shRNA<sub>mut</sub> constructs (Open Biosystems). After 24 hours, cells were trypsinized and plated sparsely on 150 mm plates in the presence of 2 ng/μl puromycin. Cells were allowed to proliferate for approximately two weeks, until colonies had formed. Colonies were picked and transferred into 24 well plates to proliferate.

Plasmids and siRNAs

The Hnrnpa1 3′ UTR (RefSeq NM_0104447) was cloned into the psiGFP-I plasmid (Stratagene) after PCR amplification from an Hnrnpa1 full length I.M.A.G.E. clone (Invitrogen). The T to G mutation in the QSBE was introduced by QuickChange site directed mutagenesis (Stratagene). Sequences of all constructs were confirmed by sequencing. tdTomato was provided by Roger Tsien and colleagues [63]. To generate a construct for expressing tdTomato in mammalian cells, the tdTomato coding sequence was excised from the original plasmid and cloned into the pcDNA 3.1+ expression vector (Invitrogen). The vector psiCheck 2 was a gift from Phil Zamore. The Hnrnpa1 wild type and mutant UTRs were cloned from the GFP expression vectors into psiCheck2.

O<sub>q</sub> siRNA1, Hnrnpa1 siRNA, and the non-targeting control siRNA were commercially designed (Ambion). The O<sub>q</sub> pGIPZ shRNA<sub>mut</sub> (Open Biosystems) was obtained from the University of Massachusetts RNAi core facility.

Reporter assays

For the fluorescent reporters, CG-4 cells were seeded onto the surface of a 24 well plate and, after 24 hours, transfected with 0.6 μg of the plasmid encoding GFP with or without a UTR of interest and 0.1 μg of the tdTomato control plasmid using Lipofectamine 2000 (Invitrogen). Complexes were formed in medium without antibiotic or other supplements. Transfections were done in DMEM containing N1 supplements, biotin, insulin, L-glutamine, but without antibiotic or B104 conditioned medium. After 24–48 hours of incubation at 37°C, the plate was read using a Victor3 plate reader (Perkin Elmer). GFP was excited using a 485 nm filter and detected through a 519/20 filter. tdTomato was excited using a 531/25 nm filter and detected through a 580/10 filter. All filters were purchased from Chroma. Each well was read 25 times, in a 5 by 5 grid with 0.1 mm spacing between the spots. The well average was computed from these 25 spots. GFP fluorescence was divided by tdTomato fluorescence to control for transfection efficiency and cell density. Three or four independently transfected wells were averaged for each experiment and the standard deviation was calculated.

For the luciferase reporters, cells were transfected as above with the plasmid psiCheck2 (Promega) or a cloned psiCheck2 variant with the hnRNP A1 wild type or T to G mutant 3′ UTR cloned behind Renilla. 24–48 hours after transfection, cells were lysed in 150 μl of lysis buffer per well and dual luciferase assays were conducted using the Dual Luciferase Assay System (Promega) according to the manufacturer’s instructions.

RNA binding assays

For the gel shift assays, recombinant QK was purified as previously described [25]. RNA gel shifts and fluorescence polarization assays were performed according to [64], with the exception that electrophoresis was carried out in a non-denaturing 5% polyacrylamide slab gel in 1xTBE buffer. RNA co-IPs from uncrosslinked lysates were conducted according to Tenenbaum et al. [65], using brains from C57BL/6 mice. Formaldehyde crosslinking of mouse brain tissue and immunoprecipitation from crosslinked lysate was conducted as described in [66]. After cross-link reversal, RNA was extracted with Trizol reagent according to the manufacturer’s instructions, the purified RNA was treated with DNase (Ambion Turbo DNase), and co-precipitated RNA was amplified by one step RT-PCR (Invitrogen) with transcript specific primers. Antibodies to QK (Bethyl labs) and 6xHisG (Invitrogen) were commercially obtained. PCR primers were fluorescently labeled at the 5′ end unless otherwise noted. After separating the PCR product from free primers by agarose gel electrophoresis, gels were imaged on a Fuji FLA-5000 using a blue laser.

Quantitative PCR

RNA was extracted from cultured cells with Trizol Reagent (Invitrogen) and treated with Turbo DNAfree (Ambion) or RQ1 DNase (Promega) according to the provided instructions. The yield was determined by spectrophotometry. Real time RT-PCR was performed using a one-step RT-PCR kit (Qiagen or Bio-Rad) according to the manufacturer’s instructions and cycled on an Opticon thermal cycler (Bio-Rad). All assays were performed in triplicate. For each target the presence of a single product after cycling was confirmed by agarose gel electrophoresis. Data were analyzed by comparison to a 5-point standard curve constructed at the time of cycling or according to the method of Rutteridge [67]. GAPDH or tubulin and tdTomato were used for normalization as indicated in the figure legends.

Splicing assays

CG-4 cells were transfected as for the dual color fluorescence reporter assay, using 20 nM siRNA and 0.3 μg GFP-selection plasmid per well. After 24 hours, the medium was replaced with 30% B104 conditioned medium containing 1500 ng/μl Geneticin (Invitrogen). Cells were incubated in the selection medium for 48 hours, then washed two times with PBS. RNA was isolated with Trizol (Invitrogen). PCR was conducted with 5′ fluorescein labeled primers and the products were isolated on a non-denaturing 5% polyacrylamide gel. Gels were imaged on a Fuji FLA-5000 imager using a blue laser. Band intensity in each lane was quantified using ImageGauge profile analysis.
Microarrays
CG-4 cells were grown and transfected as described above. RNA was extracted in Trizol (Invitrogen). RNA was further purified and genomic DNA was eliminated using the RNeasy Plus mini kit (Qiagen). Knockdown of Qk or Hnmpa1 was verified by quantitative RT-PCR. Rat Exon 1.0 ST microarrays were obtained from Affymetrix. Three independent biological replicates were assayed per group. Sample labeling and array hybridization were conducted by the University of Massachusetts Genomics Core Facility according to Affymetrix procedures. Data were analyzed with Partek GS, using the Affymetrix extended probedest annotation with a GC background correction RNA algorithm. Differentially expressed mRNAs and probesets were identified using a two-sample t-test, with an FDR of 0.1 unless otherwise noted. For analysis of the Hnmpa1 knockdown groups, probesets and transcripts that significantly changed in response to QK depletion were selected, then the changes in expression in response to hnRNP A1 depletion were clustered alongside the other control and experimental groups.

Statistical analyses
All assays were performed in triplicate unless otherwise indicated. Error bars represent the standard deviation of the experimental replicates. P-values were calculated from a two-sample t-test assuming unequal variance. Errors were propagated from the individual standard deviations according to the formula \[ \Delta Z = Z \times \sqrt{\left(\frac{A}{A^2}\right)^2 + \left(\frac{B}{B^2}\right)} \] where \( Z = A/B \).

5mer enrichment analysis
The sequences of regulated exons were obtained from Affymetrix. Exons from all rat genes were obtained from the UCSC genome browser. The frequency of 5mers in each exon was determined using Perl scripts. Random sets of exons were selected using Perl scripts. The average and standard deviation of 5mer frequency were calculated for the random sets of exons to obtain a p-value for fold enrichment significance.

Supporting Information
Figure S1 Two color fluorescence reporter assay. (A) CG-4 cells transfected with GFP, tdTomato, or co-transfected with both could be distinguished based upon red and green fluorescence. (B) CG-4 cells co-transfected with GFP and tdTomato plasmids exhibited both red and green fluorescence. (C) Red and green fluorescence could be distinguished with virtually no bleed through between the fluorescence channels.

Figure S2 QK does not interact with the Mag QASE or the Pip1 ESE. (A) The Mag intron 12 QASE and the Pip1 exon 3 ESE do not contain the high affinity QK binding element ACUAA or the related sequence AUUAA. (B) The Mag QASE and the Pip1 ESE do not bind QK with high affinity in vitro. Three overlapping fragments of the QASE or the ESE sequence, diagrammed in (A), were equilibrated with recombinant QK in vitro and analyzed by electromobility shift assays. The positive control Mbp QSBE fragment does interact with QK.

Figure S3 QK co-precipitates with Hnmpa1 mRNA but not other splicing factors. Formaldehyde crosslinked mouse brain lysate was immunoprecipitated with QK or control His6G antibodies. Co-precipitated RNA was purified and specific RNAs were amplified by RT-PCR followed by separation on an agarose gel. Lanes are as indicated. Note that the Sfps5 and Hnmpa1 gel images are the same as those presented in Figure 2.

Figure S4 QK but not SFRS5 or hnRNP C regulates Mag exon 12 inclusion. The isoform ratio of Mag was assessed as in Figure 5. Transfection with two different Sfps5-targeted siRNAs or two different Hnmpa1-targeted siRNAs does not appreciably alter Mag isoform ratio relative to control siRNA or a GFP transfection control. In contrast, transfection of a Qk targeting siRNA increases Mag exon 12 inclusion by two-fold.

Figure S5 Correlation coefficient as a function of false discovery rate. Microarray probeset expression values were analyzed as described in the text, for four different False Discovery Rates. Briefly, probesets with changes in expression in both of the Qk knockdown groups were selected at the desired FDR. Expression values for the three biological replicates were averaged and the difference from the control CG-4 value was calculated. Correlations between the changes in expression for the indicated groups were calculated using Partek GS Software.

Figure S6 Correlation between Hnmpa1 knockdown and Qk knockdown samples at the transcript level. QK-dependent transcript expression changes are correlated with transcript expression changes caused by hnRNP A1 depletion. Transcripts with expression changes in response to Qk knockdown were identified using a two sample t-test, comparing the knockdown groups to the control groups. The mean of each group was determined and the difference from the CG-4 cell line was calculated. Values were clustered as described for Figure 7 for probesets.

Table S1 Transcripts significantly changed in response to QK knockdown in CG-4 oligodendrocyte precursor cells.

Table S2 Enriched 5mers in alternatively spliced exons.

Table S3 Transcripts found to decrease upon QK depletion and containing a QSBE within their 3’ UTR.

Table S4 Genes implicated in SCZ that change upon QK depletion.

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Author Contributions
Conceived and designed the experiments: NRZ SPR. Performed the experiments: NRZ CCC LMM. Analyzed the data: NRZ CCC BMF SPR. Contributed reagents/materials/analysis tools: NRZ CCC BMF LMM. Wrote the paper: NRZ SPR.
References

1. Lappe-Siefke C, Geobaldo S, Gravel M, Nicksh E, Lee J, et al. (2003) Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nat Genet 33: 366-374.

2. Gabriel H, Klaugmann M, Anderson T, Vool D, Thomson C, et al. (1998) Axonal swellings and degeneration in mice lacking the major proteolipid of myelin, Science 280: 1610-1613.

3. Li C, Tropak MB, Gerlai R, Clapoff S, Abravanel-Newly W, et al. (1994) Myelination in the absence of myelin-associated glycoprotein. Nature 369: 747-750.

4. Vernet C, Arzt K (1997) STAR, a gene family involved in signal transduction and activation of RNA. Trends Genet 13: 479-484.

5. Sidman RL, Dickie MM, Appel SH (1964) Mutant Mice (Quaking and Jimpy) with Deficient Myelination in the Central Nervous System. Science 144: 309-311.

6. Ebersole T, Rho O, Arzt K (1992) The proximal end of mouse chromosome 17: new noncoding markers identify a deletion associated with quaking-like. Genetics 131: 183-190.

7. Hardy RJ, Loshin CI, Friedrich VL, Jr., Chen Q, Ebersole TA, et al. (1996) Neural cell type-specific expression of QKI proteins is altered in quaking-like mutant mice. J Neurosci 16: 7941-7949.

8. Zhao L, Tian D, Xia M, Mackin WB, Feng Y (2006) Rescuing qkV dysmyelination by a single isoform of the selective RNA-binding protein QKI. J Neurosci 26: 11276-11286.

9. Neurotrophic factor Hardy R, Hopfer JD, Vogel H, Justice MJ (2005) A new ENU-induced allele of mouse quaking causes severe CNS dysmyelination. Manm Genome 16: 672-682.

10. Li Z, Zhang Y, Li D, Feng Y (2000) Destabilization and mislocalization of myelin basic protein mRNAs in quaking dysmyelinating lacking the QKI RNA binding proteins promotes oligodendroglial differentiation. Nat Neurosci 3: 27-33.

11. Zhao L, Ku L, Chen Y, Xia M, LePresti P, et al. (2006) QKI binds MAP1B mRNA and enhances MAP1B expression during oligodendrocyte development. Mol Biol Cell 17: 4179-4186.

12. Leducque D, Galarneau A, Liu HN, Scott M, Almazan G, et al. (2005) Protection of p27Kip1 mRNA by quaking RNA binding proteins promotes oligodendrocyte differentiation. Nat Neurosci 8: 1813-1820.

13. Wu JI, Reed RB, Grabowski PJ, Artzt K (2002) Function of quaking in myelin formation. Biochim Biophys Acta 1779: 486-494.

14. Wu JI, Reed RB, Grabowski PJ, Artzt K (2002) Function of quaking in myelination: regulation of alternative splicing. Proc Natl Acad Sci U S A 99: 4164-4169.

15. Aberg K, Saetre P, Lindholm E, Ekholm B, Pettersson U, et al. (2006) Human relaxation of MBP mRNAs in the quaking viable mice. Neuron 36: 815-829.

16. Zhou W, Rolish ME, Yeo G, Tung V, Mawson M, et al. (2004) Systematic analysis of regulated RNA networks in the brain. Science 302: 1212-1215.

17. McCullumsmith RE, Gupta D, Beneyto M, Kreger E, Haroutunian V, et al. (2006) Human QKI, a gene with a severe dysmyelinating phenotype: downregulated in multiple brain regions in schizophrenia. Proc Natl Acad Sci U S A 103: 7482-7487.

18. Haroutunian V, Katsel P, Dracheva S, Davis KL (2006) The human homolog of the Quaking STAR protein. Nat Struct Mol Biol 12: 691-698.

19. Schmauss C (1996) Enhanced cleavage of an atypical intron of dopamine D3-receptor pre-mRNA in quaking dysmyelination lacking the QKI RNA binding proteins promotes oligodendrocyte differentiation. Nat Neurosci 3: 27-33.

20. Zhang Y, Bani MR, Lu S, Rowan S, Ben-David Y, et al. (1994) The A1 and A1B proteins of heterogeneous nuclear ribonucleoprotein regulate 5’ splice site selection in the hnRNPA1/A2 binding sites control. Mol Biol Cell 17: 4179-4186.

21. Schmauss C (1996) Enhanced cleavage of an atypical intron of dopamine D3-receptor pre-mRNA in quaking dysmyelination lacking the QKI RNA binding proteins promotes oligodendrocyte differentiation. Nat Neurosci 3: 27-33.

22. Umemori H, Kadowaki Y, Hirosawa K, Yoshida Y, Hironaka K, et al. (1999) Akt pathways: regulation of alternative splicing in vivo. P Natl Acad Sci U S A 96: 6924-6928.

23. Zhang Y, Bani MR, Lu S, Rowan S, Ben-David Y, et al. (1994) The A1 and A1B proteins of heterogeneous nuclear ribonucleoprotein promote alternative splicing in vivo by overexpression of antigenic splicing factors. Cell 84: 1706-1709.

24. Han K, Yeo G, An P, B urge CB, Grabowski PJ (2005) A combinatorial code for splicing regulation of pre-mRNA splicing by hnRNPA1 and splicing factor SF2. Cell 68: 365-375.

25. Avila CJ, Stanssens S, Hellemans DM, Kraemer AR (1994) Regulation of alternative splicing in vivo by overexpression of antigenic splicing factors. Science 265: 1706-1709.

26. Zhang Y, Bani MR, Lu S, Rowan S, Ben-David Y, et al. (1994) The A1 and A1B proteins of heterogeneous nuclear ribonucleoprotein promote alternative splicing in vivo. P Natl Acad Sci U S A 96: 6924-6928.

27. Burd GD, Dreyfuss G (1994) RNA binding specificity of the hnRNP A1: significance of the A1/A2 affinity binding sites in pre-mRNA splicing. Embryol J 13: 1197-1204.

28. Zhang Y, Bani MR, Lu S, Rowan S, Ben-David Y, et al. (1994) The A1 and A1B proteins of heterogeneous nuclear ribonucleoprotein promote alternative splicing in vivo. P Natl Acad Sci U S A 96: 6924-6928.

29. Burd GD, Dreyfuss G (1994) RNA binding specificity of the hnRNP A1: significance of the A1/A2 affinity binding sites in pre-mRNA splicing. Embryol J 13: 1197-1204.

30. Han K, Yeo G, An P, B urge CB, Grabowski PJ (2005) A combinatorial code for splicing regulation of pre-mRNA splicing by hnRNPA1 and splicing factor SF2. Cell 68: 365-375.
60. Stefansson H, Rujescu D, Cichon S, Pietilainen OP, Ingason A, et al. (2008) Large recurrent microdeletions associated with schizophrenia. Nature 455: 232–236.

61. McCarthy SE, Makarov V, Kirov G, Addington AM, McClellan J, et al. (2009) Microduplications of 16p11.2 are associated with schizophrenia. Nat Genet 41: 1223–1227.

62. Zhang F, Seeman P, Liu P, Weterman MA, Gonzaga-Jauregui C, et al. (2010) Mechanisms for nonrecurrent genomic rearrangements associated with CMT1A or HNPP: rare CNVs as a cause for missing heritability. Am J Hum Genet 86: 892–903.

63. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, et al. (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 22: 1567–1572.

64. Pagano JM, Farley BM, McCoig LM, Ryder SP (2007) Molecular basis of RNA recognition by the embryonic polarity determinant MEX-5. J Biol Chem 282: 8883–8894.

65. Tenenbaum SA, Lager PJ, Carson CC, Keene JD (2002) Ribonomics: identifying mRNA subsets in mRNP complexes using antibodies to RNA-binding proteins and genomic arrays. Methods 26: 191–198.

66. Chen-Plotkin AS, Sadri-Vakili G, Yohrling GJ, Braverman MW, Benn CL, et al. (2006) Decreased association of the transcription factor Sp1 with genes downregulated in Huntington’s disease. Neurobiol Dis 22: 233–241.

67. Rutledge RG (2004) Sigmoidal curve-fitting redifines quantitative real-time PCR with the prospective of developing automated high-throughput applications. Nucleic Acids Res 32: e178.