RNA-binding Protein Quaking Stabilizes Sirt2 mRNA during Oligodendroglial Differentiation

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Myelination is controlled by timely expression of genes involved in the differentiation of oligodendrocyte precursor cells (OPCs) into myelinating oligodendrocytes (OLs). Sirtuin 2 (SIRT2), a NAD⁺-dependent deacetylase, plays a critical role in OL differentiation by promoting both arborization and downstream expression of myelin-specific genes. However, the mechanisms involved in regulating SIRT2 expression during OL development are largely unknown. The RNA-binding protein quaking (QKI) plays an important role in myelination by post-transcriptionally regulating the expression of several myelin-specific genes. In quaking viable (qkv/qkv) mutant mice, SIRT2 protein is severely reduced; however, it is not known whether these genes interact to regulate OL differentiation. Here, we report for the first time that QKI directly binds to Sirt2 mRNA via a common quaking response element (QRE) located in the 3' untranslated region (UTR) to control SIRT2 expression in OL lineage cells. This interaction is associated with increased stability and longer half-lives of Sirt2.1 and Sirt2.2 transcripts leading to increased accumulation of Sirt2 transcripts. Consistent with this, overexpression of qki promoted the expression of Sirt2 mRNA and protein. However, overexpression of the nuclear isoform qkI-5 promoted the expression of Sirt2 mRNA, but not SIRT2 protein, and delayed OL differentiation. These results suggest that the balance in the subcellular distribution and temporal expression of QKI isoforms control the availability of Sirt2 mRNA for translation. Collectively, our study demonstrates that QKI directly plays a crucial role in the post-transcriptional regulation and expression of Sirt2 to facilitate OL differentiation.

This manuscript is dedicated in memory of our colleague Dr. J. Ronald Doucette.

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4 The abbreviations used are: OL, oligodendrocyte; OPC, oligodendrocyte precursor cells; V2/SVZ, ventricular/subventricular zone; SIRT2, Siruin2; QKI, quaking; QRE, quaking response element; PLP, proteolipid protein; GM, growth medium; co-IP, co-immunoprecipitation; DM, differentiation medium; OGM, OL growth medium; qRT, quantitative real time; MBP, myelin basic protein; Aip-1, actin interacting protein 1; Map1B, microtubule-associated protein 1B (Map1B) (22), and heterologous nuclear ribonucleoprotein A1 (hnRNPA1) (23). This interaction alters the stability of the target mRNAs, which can lead to either a promotion or inhibition in the downstream translation of the protein products, as is the case for p27kip1 (20) or Aip-1 (21), respectively. In addition to stabilizing the target mRNA, QKI has been demonstrated to regulate alternative splicing of Mag (8, 10), and an altered...
ratio of splice variants for Mag (24) and Mbp (7) are found in the qk⁺/qk⁺ mutants. qk⁺/qk⁻ mice also show a mislocalized pattern of Mbp, with it being confined to the nucleosis (6, 7). Thus, QKI proteins interact with target transcripts at multiple levels to control gene expression during oligodendroglial differentiation.

Similar to myelin structural proteins (MBP and PLP), SIRT2 protein expression is also reduced in qk⁺/qk⁻ mice (25). Mammalian SIRT2 is a class III NAD⁺-dependent deacetylase (26), which is predicted to give rise to three isoforms (27). SIRT2 is enriched in brain and spinal cord tissue, predominately localized in the paranodal regions of the CNS myelin sheath (28–30). During OL differentiation, SIRT2 is expressed early, prior to the expression of myelin-specific genes (25, 30, 31), promoting differentiation at both the cellular and molecular level (31). In addition to the qk⁺/qk⁻ mice, SIRT2 expression is reduced in the Plp null mice (29) and Plp-ISEdel mutant mice (25). In Plp mutants, the loss of SIRT2 is observed primarily in the myelin sheath (25, 29) but not in OPCs or OL cell bodies (25). Moreover, no putative QREs have previously been identified in Sirt2 mRNA. Thus, it was postulated that QKI indirectly regulates SIRT2 expression during CNS myelination through co-transport with PLP into the myelin sheath (25).

To further investigate the relationship between qki and Sirt2 in OL development, we used mouse primary OLs and the CG4-OL cell line derived from neonatal rat forebrain O-2A progenitors that undergo defined stages of differentiation under controlled media conditions (31–36). Coordinated expression of QKI and SIRT2 was observed during differentiation. Thus, we sought to delineate the molecular mechanisms that govern the direct or indirect interaction between the RNA-binding protein QKI and Sirt2 during OL development. We demonstrate a direct interaction between QKI and Sirt2 mRNA in OL progenitors and differentiating OLs. The binding site for QKI was mapped to the QRE ACUAAC at 1853–1858 bp in the 3’ UTR of Sirt2 mRNA. Our findings indicate that Sirt2 is a direct target of QKI. Binding of QKI increases the post-transcriptional stability of Sirt2 mRNA and controls its availability for translation. Furthermore, our results show QKI-5 delays the transition from OPC through to post-mitotic, immature OL resulting in a delay in differentiation. The subcellular localization and coordinated temporal expression of specific QKI isoforms appear to govern Sirt2 expression for proper OL differentiation.

Results

Expression of QKI and SIRT2 Increase during OL Differentiation—QKI plays a critical role in OL differentiation by regulating the expression of several myelin specific genes, such as Mbp (6, 7), Plp (9), and Mag (8, 10, 42). We have previously demonstrated that SIRT2 can promote OL differentiation (31). Hence, we sought to delineate the molecular interaction between qki and Sirt2. In CG4-OL cells, the expression pattern of both qki (Fig. 1) and Sirt2 (Fig. 2) increased over 6 days of differentiation. Detailed quantitative mRNA and protein analyses revealed differential expression of specific isoforms of QKI and SIRT2 during OL differentiation in vitro. Expression of qki mRNA (Fig. 1A) gradually increased with differentiation reaching a ~3-fold increase by day 6. There was a corresponding increase in expression of the QKI protein product QKI-6 isoform (~2-fold increase at day 6 in differentiation medium (DM)) but not of QKI-5 or QKI-7, which co-migrate on the immunoblot (Fig. 1, B and C).

The Sirt2.2 variant was the most abundant transcript in CG4-OL cells with ~4.5-fold and ~2700-fold greater expression than Sirt2.1 and Sirt2.3, respectively, under growth conditions (Fig. 2A). Expression of the Sirt2.2 transcript increased ~8-fold within 24 h under differentiation conditions and was maintained through to day 6. Similarly, Sirt2.1 and Sirt2.3 mRNA expression increased throughout differentiation. Sirt2.2 was also the most abundant protein isoform in CG4-OL cells (Fig. 2B). Expression of SIRT2.2 protein increased ~2.5-fold by day 3 and was maintained through to day 6 (Fig. 2C). Although Sirt2.1 and Sirt2.3 mRNA increased during differentiation, this did not translate to an increase in the SIRT2.1 or SIRT2.3 protein products. The extent of differentiation was analyzed by quantifying the expression of mature OL marker MBP. Expression of Mbp mRNA (supplemental Fig. S1A) increased during differentiation reaching a ~60-fold increase by day 6. Consistent with this, expression of MBP protein (supplemental Fig. S1B) also increased with differentiation. The coordinated expression patterns of qki and Sirt2 during OL differentiation suggests that these two genes may interact, either directly or indirectly, to promote OL differentiation.

Overexpression of qki Induces the Expression of Sirt2 mRNA and Protein—Quaking viable mutant mice (qk⁺/qk⁻) exhibit reduced SIRT2 protein expression in myelin sheath (25), yet there is little known about the relationship between QKI and Sirt2 mRNA during CNS myelination. To investigate what role QKI may have in regulating the expression of Sirt2 during OL differentiation, qki was overexpressed in CG4-OL cells by transfection with the common coding sequence of the RNA binding domain from QKI-5, QKI-6, and QKI-7. Up-regulation of qki resulted in an increase in Sirt2 mRNA expression under both proliferating (growth media; GM) (Fig. 3A) and differentiating (DM, day 6) conditions (Fig. 3B). In GM, overexpression of qki in proliferating CG4-OLs increased Sirt2.1 and Sirt2.2 mRNA levels to ~2- and ~3-fold, respectively; in contrast, Sirt2.3 expression was not affected (Fig. 3A). In DM, expression of all three variants increased ~2-fold in differentiating CG4-OLs (Fig. 3B). Up-regulation of qki also resulted in a corresponding increase in Sirt2.1 and Sirt2.2 protein expression under both proliferating (Fig. 3, C and D) and differentiating (Fig. 3, E and F) conditions in CG4-OLs. Furthermore, to examine the regulatory function of QKI in OLs, qki was overexpressed in primary OLs isolated from the mouse brain (35, 36). Similar to CG4-OLs, overexpression of qki in mouse primary OLs increased Sirt2 mRNA expression under both proliferating (GM) (Fig. 4A) and differentiating (DM, day 6) conditions (Fig. 4B). In proliferating primary OLs, up-regulation of qki increased the Sirt2.1 and Sirt2.2 mRNA expression ~2-fold; whereas Sirt2.3 expression was not altered (Fig. 4A). In DM, expression of all three variants increased ~3-fold in differentiating primary OLs (Fig. 4B). There was a corresponding increase in Sirt2.1 and Sirt2.2 protein expression under both proliferating (Fig. 4, C and D) and differentiating (Fig. 4, E and
(F) conditions in primary OLs. Under these experimental conditions, expression of the SIRT2.3 protein isoform was not detectable either with or without qkI overexpression (Figs. 3, C and E, and 4, C and E). Thus, qkI appears to be a positive regulator of Sirt2.1 and Sirt2.2 mRNA and protein expression in developing OLs.

Presence of Putative QREs in Sirt2 Transcripts—SIRT2 protein expression is reduced in both qkv/qkv (25) and Plp (25, 29) mutant mice, and the expression of PLP is critical for the transport of SIRT2.2 to the myelin sheath (25, 29). Thus, it is not known whether QKI controls the expression of SIRT2 during CNS myelination through a direct interaction with Sirt2 mRNA or through an indirect pathway involving PLP. Using in silico analysis, we identified the presence of two putative QREs in Sirt2 mRNA in the 3′ UTR (Fig. 5A). Both putative QREs, AUUAA(C/U) at 1639–1643 bp (denoted as QRE-1) and ACUAA(C/U) at 1853–1858 bp (denoted as QRE-2) (Fig. 5A) in the Sirt2 mRNA, correspond to the predicted consensus sequences (8, 18) and are highly conserved (Fig. 5A). Thus, the 3′ UTR of Sirt2 mRNA has two putative interaction sites for QKI.

QKI Binds to Sirt2 mRNA to Regulate Its Expression—To determine whether QKI binds to Sirt2 mRNA at the putative binding sites, RNA co-immunoprecipitation (RNA co-IP) was carried out using whole cell lysates from CG4-OL under both
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FIGURE 2. Expression of Sirt2 mRNA and protein increases during differentiation. CG4-OL progenitor cells in GM were transferred to DM and whole cell lysates were collected at 24-h intervals (days 0, 1, 2, 3, 4, 5, and 6). A, Sirt2 mRNA expression increases under differentiation conditions. Sirt2.2 mRNA is the most abundant transcript expressed in CG4-OL cells and increases by day 1 of differentiation. qRT-PCR data (n = 3 biological replicates) were normalized to β-actin and represented relative to Sirt2.3 variant at day 0 (mean ± S.E.; two-way ANOVA). ‡ denotes Sirt2.1 mRNA with p < 0.001; † denotes Sirt2.3 mRNA with p < 0.001 relative to their respective variants at day 0. B, representative immunoblot showing a corresponding increase in SIRT2 protein expression over the course of differentiation. C, densitometric analysis of immunoblots (n = 3 biological replicates) reveal that SIRT2.2 is the predominate isoform expressed during OL differentiation. Data were normalized to β-tubulin and represented relative to the respective isoform at day 0 (mean ± S.E.; two-way ANOVA, ***, p < 0.001).

growth (GM) and differentiating (DM day 6) conditions. QKI was found to bind Sirt2 mRNA in both proliferating and differentiating CG4-OLs (Fig. 5, B and C). In comparison, QKI was found to bind Plp mRNA only under growth conditions (supplemental Fig. S3A). In both GM and DM, QKI did not bind with Gapdh mRNA (Fig. 5B, bottom panel). Further investigation determined that under growth conditions QKI bound to Sirt2.1 and Sirt2.2 only in CG4-OLs (Fig. 5D), whereas under differentiating conditions QKI bound to all the three variants of Sirt2 mRNA (Fig. 5E). In addition, interaction of QKI and Sirt2 mRNA in primary OLs was also examined. QKI was found to interact with Sirt2 mRNA in both proliferating and differentiating primary OLs (Fig. 6A). In proliferating primary OLs under growth conditions, QKI bound to Sirt2.1 and Sirt2.2 mRNA
whereas QKI bound to all the three variants of Sirt2 in differentiating primary OLs (Fig. 6C). Collectively, these data demonstrate that QKI interacts with all three variants of Sirt2 mRNA, presumably via the putative QRE-1 and/or QRE-2 in the 3′/H11032 UTR.

QKI Binds to 3′/H11032 UTR of Sirt2 mRNA—To identify the critical QRE site(s) involved in QKI-Sirt2 interaction, RNA co-IP and luciferase reporter assay systems were used. RNA co-IP was performed with wild-type or mutated putative QREs of the Sirt2 3′/H11032 UTR transfected in HEK293 cells with or without qkI. The QRE mutations were as follows (Fig. 7A): (i) Sirt2 3′ UTR-Mut1 for QRE-1 at 1639 bp (AUUAAC→UCGACC), (ii) Sirt2 3′ UTR-Mut2 for QRE-2 at 1853 bp (ACUAAC→UCGAGC), and (iii) Sirt2 3′ UTR-Mut1*Mut2 for combined QRE-1 and QRE-2 mutations. RNA co-IP revealed that QKI binds to wild-type and mutated QRE-1 (Sirt2 3′/H11032 UTR-Mut1) of Sirt2 mRNA, whereas mutation of QRE-2 (Sirt2 3′ UTR-Mut2 and Sirt2 3′ UTR-Mut1*Mut2) prevented binding (Fig. 7B). Luciferase reporter assay was also carried out using wild-type or mutated QREs of the Sirt2 3′/H11032 UTR cloned downstream to the firefly luciferase, co-transfected in CG4-OL cells together with qkI. Overexpression of qkI resulted in an increased luciferase activity of Sirt2 3′/H11032 UTR (Fig. 7C). Mutation of QRE-1 (Sirt2 3′ UTR-Mut1) also resulted in increased luciferase activity indicating QKI was still able to interact with Sirt2 3′ UTR despite the mutation to QRE-1. In contrast, mutation of QRE-2 either alone (Sirt2 3′ UTR-Mut2) or double mutation in QRE-1/QRE-2 (Sirt2 3′ UTR-Mut1*Mut2) failed to increase the luciferase activity.
erase activity. These results indicate that the nucleotides in QRE-2 (1853–1858 bp) are critical for the functional interaction between QKI and Sirt2. Thus, QKI binding to Sirt2 in the QRE ACUAAC (1853–1858 bp) appears to be important for regulating the expression of Sirt2 during OL development.

QKI Stabilizes and Protects Sirt2 mRNA from Degradation—QKI regulates myelin gene expression during development, in part by modulating the stability of myelin gene transcripts. To determine whether this is also the case with Sirt2, a standard mRNA stability assay (20–22) was used to assess the ability of QKI to protect or degrade Sirt2 transcripts. Following transfection with pcDNA or pcDNA-qkI (48 h), actinomycin D treatment was used to inhibit transcription under GM conditions and subsequent degradation of Sirt2 mRNA was monitored using qRT-PCR. QKI was found to regulate the stability of Sirt2.1 and Sirt2.2 variants (Fig. 8, A–C). Overexpression of qkI delayed the degradation of Sirt2.1 (Fig. 8A) and Sirt2.2 (Fig. 8B), but not Sirt2.3 (Fig. 8C). The half-life (t₁/₂) of each of the Sirt2

FIGURE 4. QKI promotes the expression of Sirt2 mRNA and protein in primary OLs. Primary OLs were electroporated with a pcDNA vector containing qkI cDNA (common coding region of qkI-5, qkI-6, and qkI-7, see “Materials and Methods”) and cultured under both growth (GM) and differentiation (DM) conditions for 6 days after electroporation. Up-regulation of qkI increases the expression of Sirt2.1 and Sirt2.2 mRNA in GM (A), whereas all three variants of Sirt2 transcripts (Sirt2.1, Sirt2.2, and Sirt2.3) were increased in DM (B). qRT-PCR data (n = 3 biological replicates) was normalized to β-actin and represented relative to pcDNA control vector (mean ± S.E.; two-way ANOVA, *, p < 0.05; ***, p < 0.001). Representative immunoblots (C and E) and densitometric analysis (D and F) shows a corresponding increase in SIRT2.1 and SIRT2.2 protein with the overexpression of qkI. SIRT2.3 protein was below the limits of detection. Densitometry data (n = 3 biological replicates) were normalized to β-tubulin and represented relative to pcDNA control vector (mean ± S.E.; two-way ANOVA, *, p < 0.05; ***, p < 0.001).
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A

Sirt2 mRNA

5' UTR

Coding DNA Sequence

3' UTR

1639 - 1643

QRE-1

1853 - 1858

QRE-2

Mouse Sirt2 3'UTR

GGCUAAUCAACUACCCCTCGAUAAGGGGCGCUCCAAUAUGAA

1679

Rat Sirt2 3'UTR

AGCUAAUCAAAGGUCUACCCCTCGAUAAGGGGCGCUCCAAUAUGAA

1885

Human Sirt2 3'UTR

UAAGUAAACCAACCUACCCAGUGUGGUG-UGGGC-CUCUGAAUAA

1902

QRE-2

Mouse Sirt2 3'UTR

CCUUGCUCUAAUAAGAACAACAAACAAAACAAACAAACACG

1863

Rat Sirt2 3'UTR

GCAAAGUUGAUUAGAAACAAUAACACUGACCAAAAACACG

2059

Human Sirt2 3'UTR

CAUUGCUAAUGAGAACAACAAACAAAACAAACACG

2079

B

GM

Input Anti-IgG Anti-QKI

226bp

452bp

DM

Input Anti-IgG Anti-QKI

Sirt2

 anti-QKI

Gapdh

C

Relative Quantification of Sirt2 mRNA bound to QKI protein in CCG-OLs

Anti-IgG Anti-QKI

***

D

Relative quantification of Sirt2.1, Sirt2.2 and Sirt2.3 mRNA in CCG-OLs

Sirt2.1 Sirt2.2 Sirt2.3

Anti-IgG GM Anti-QKI GM

***

E

Relative quantification of Sirt2.1, Sirt2.2 and Sirt2.3 mRNA variants bound to QKI protein in CCG-OLs

Sirt2.1 Sirt2.2 Sirt2.3

Anti-IgG DM Anti-QKI DM

***
variants were calculated (39, 40) by measuring the reduction in the mRNA levels to 50% from their respective initial mRNA level. Sirt2.1, Sirt2.2, and Sirt2.3 mRNA $t_{1/2}$ were determined to be 42.5 ± 8.6, 23.8 ± 2.8, and 62.8 ± 7.4 min in CG4-OLs transfected with blank control vector (Fig. 8, A–D) and 78.7 ± 3.5, 66.8 ± 1.2, and 50.9 ± 2.9 min in CG4-OL cells transfected with $qkI$, respectively (Fig. 8, A–D). Hence, there was ~1.8- and ~2.8-fold increase in the $t_{1/2}$ of Sirt2.1 and Sirt2.2 transcripts with $qkI$ overexpression, but little difference in the $t_{1/2}$ of Sirt2.3 mRNA. Sirt2.2 appears to be the primary functional target of $qkI$, as >85% of the mRNA transcripts remain protected 30 min after transcription is blocked in CG4-OL cells transfected with $qkI$ (Fig. 8B) compared with <40% of mRNA transcripts remaining in cells transfected with control vector. Our finding suggests that binding of $qkI$ to Sirt2.1 and Sirt2.2 mRNA increases their stabilization and in turn may promote SIRT2.1 and SIRT2.2 protein expression during OL development.

Overexpression of $qkI$-5 Induces the Expression of Sirt2 mRNA but Not SIRT2 Protein—There is a wealth of data supporting the role of $qkI$-6 in promoting OL differentiation and myelination (5, 9, 10, 13, 20–22), yet the role of $qkI$-5 is not well defined (5, 6). Although $qkI$ expression increased gradually with differentiation (Fig. 1), this was found to reflect an increase in $qkI$-5 and $qkI$-6 transcripts but no change in $qkI$-7 expression (Fig. 9A). $qkI$-5 mRNA is expressed early during differentiation, with a ~1.5-fold increase by day 3 and ~2-fold increase by day 6, whereas $qkI$-6 mRNA expression increased ~1.5-fold by day 6 in DM. $qkI$-5 protein expression did not show a significant increase during differentiation most likely due to the co-migration of $qkI$-5 and $qkI$-7 (Fig. 1, B and C).

FIGURE 5. Putative binding sites of $qkI$ protein in the Sirt2 mRNA. A, a schematic diagram and sequence alignment showing the positions of predicted QRE, denoted as QRE-1 at 1639–1643 bp and QRE-2 at 1853–1858 bp, in the 3′ UTR of mouse Sirt2 mRNA. Sequence alignment of the mouse, rat, and human Sirt2 mRNA 3′ UTR reveals highly conserved residues for both the QRE-1 and QRE-2 regions (black boxes). B and C, RNA co-immunoprecipitation with anti-$qkI$ antibody using the whole cell lysate from CG4-OL cells show binding of $qkI$ to Sirt2 mRNA under both growth (GM) and differentiation (DM) conditions. B, RT-PCR products of Sirt2 and Gapdh were detected in a RedSafe-stained agarose gel; lanes are as indicated. C, qRT-PCR data ($n = 3$ biological replicates) was normalized to $β$-actin and represented relative to control IgG (mean ± S.E.; two-way ANOVA, ***,$p < 0.001$). D and E, $qkI$ binds to Sirt2.1 mRNA and Sirt2.2 mRNA under both growth (GM) (D) and differentiation (DM) (E) conditions, whereas binding of $qkI$ to Sirt2.3 mRNA was only observed under DM conditions in CG4-OLs (E). qRT-PCR data ($n = 3$ biological replicates) were normalized to $β$-actin and represented relative to control IgG (mean ± S.E.; two-way ANOVA, ***,$p < 0.001$).

FIGURE 6. $qkI$ interacts with Sirt2 mRNA in primary OLs. A, RNA co-immunoprecipitation with anti-$qkI$ antibody using the whole cell lysate from primary OLs shows binding of $qkI$ to Sirt2 mRNA under both growth (GM) and differentiation (DM) conditions. qRT-PCR data ($n = 3$ biological replicates) were normalized to $β$-actin and represented relative to control IgG (mean ± S.E.; two-way ANOVA, ***,$p < 0.001$). B, in GM, $qkI$ was bound only to Sirt2.1 mRNA and Sirt2.2 mRNA. C, in DM, $qkI$ was bound to all the three variants of Sirt2 mRNA, qRT-PCR data ($n = 3$ biological replicates) were normalized to $β$-actin and represented relative to control IgG (mean ± S.E.; two-way ANOVA, ***,$p < 0.001$).
As Sirt2 mRNA expression increases early in OL differentiation (Fig. 2), we chose to further investigate the specific role of QKI-5 in regulating Sirt2 expression. Overexpression of qkI-5 induced expression of qkI-5 transcript only and did not alter qkI-6 or qkI-7 mRNA levels in differentiating OLs (Fig. 9 B, supplemental Fig. S2, A–C). Up-regulation of qkI-5 resulted in an increase in the expression of Sirt2 transcripts. A ~5-fold increase in Sirt2 mRNA compared with cells transfected with control vector was observed by day 6 (Fig. 9C); however, up-regulation of qkI-5 did not increase SIRT2 protein expression (Fig. 9D). Similarly, up-regulation of qkI-5 increased Plp mRNA expression but not PLP protein expression (supplemental Fig. S3, B–D). Although QKI-5 is able to modulate transcript levels of Sirt2 and Plp in differentiating OLs, this does not result in a corresponding change in expression of the protein product.

**QKI-5 Delays CG4-OL Differentiation**—Given that QKI-5 regulates Sirt2 only at the level of mRNA expression, we sought to better understand its functional role during OL differentiation. To assess differentiation, CG4-OL cell cultures were analyzed for phenotypic markers of specific OL developmental stages at 24-h intervals (Fig. 10).

**FIGURE 7. QKI interacts with Sirt2 via the QRE (ACUAAC) at 1853 bp in the 3’ UTR.** A 593-bp fragment of the Sirt2 3’ UTR containing putative QKI binding sites was amplified and cloned into pGL3-promoter vector. A, site-directed mutagenesis of the putative QKI response elements, QRE-1 at 1639 bp (Mut1), QRE-2 at 1853 bp (Mut2), or both QREs at 1639 and 1853 bp (Mut1*Mut2) were carried out using the QuikChange Lightning kit. The underlined and bold sequences represent putative QREs and the mutated sequences represented in red. B, HEK293 cells were co-transfected with pcDNA or pcDNA-qkI (common coding region of qkI-5, qkI-6, and qkI-7) along with pGL3 vector harboring wild-type or mutated QREs in the Sirt2 3’ UTR, as indicated. RNA co-immunoprecipitation with His tag antibody in these cell extracts reveals high affinity binding of QKI to wild-type or mutated QRE-1 of Sirt2 3’ UTR but not to mutated QRE-2. qRT-PCR data (top panel) (n = 3 biological replicates) was normalized to β-actin and represented relative to pcDNA control vector (mean ± S.E.; two-way ANOVA, ***, p < 0.001). RT-PCR products (bottom panel) were detected in a RedSafe-stained agarose gel; lanes are as indicated. C, for luciferase reporter assay, CG4-OL cells were co-transfected with pcDNA or pcDNA-qkI (common coding region of qkI-5, qkI-6, and qkI-7) along with pGL3 luciferase vector containing wild-type or mutated QREs in the Sirt2 3’ UTR. Overexpression of qkI increased luciferase reporter activity of Sirt2 3’ UTR and Sirt2 3’ UTR-Mut1 but not Sirt2 3’ UTR-Mut2 or Sirt2 3’ UTR-Mut1*Mut2. This indicates that QRE-2 at 1853 bp is critical for the interaction of QKI with Sirt2. Relative luciferase activity (n = 4 biological replicates) was normalized to pcDNA control vector co-transfected with pGL3 luciferase vector and represented as percent activity (mean ± S.E.; two-way ANOVA, ***, p < 0.001).
whereas GalC^+/ve cells (pre-myelinating OLs) are post-mitotic and develop extensive branching of cellular processes (43–45). In differentiating CG4-OL cultures, ~80% of cells expressed A2B5 at day 1 under all treatment conditions. This was followed by a steady decline in A2B5^+/ve cells corresponding to an increase in GalC^+/ve cells over the 6 days of differentiation (Fig. 10, E and F). The stability of Sirt2.2 mRNA was not significantly impacted (C). A–C, qRT-PCR data (n = 3 biological replicates) were normalized to 18S rRNA and are represented as the percentage of Sirt2 mRNA variant levels measured at time 0 min (mean ± S.E.; one-phase decay). The half-lives were calculated as the time necessary for each Sirt2 mRNA variant to reduce to 50% of its initial amount at time 0 min (dashed lines). D, half-lives of Sirt2.1, Sirt2.2, and Sirt2.3 mRNA are represented in minutes (mean ± S.E.; two-way ANOVA, ***, p < 0.001).

**Discussion**

*Sirt2* is a key regulator of OL development (31). In myelinating OLs, SIRT2 is predominately cytoplasmic, localizing to the paranodal loops (28–30), but under certain cellular conditions, SIRT2 has also been shown to shuttle between the cytoplasm and nucleus (26, 46). As a NAD^+/^-dependent deacetylase, it has a multitude of cellular targets involved in gene transcription (47, 48), proliferation (46, 47, 49, 50), cell polarity (51), and cytoskeletal remodeling (26, 28, 30). We have previously shown expression of Sirt2 drives OL differentiation at the cellular level by enhancing process outgrowth and arborization (31). QKI facilitates proper CNS myelination via post-transcriptional regulation of several transcripts during OL development (5–10, 20, 22, 23). Interestingly, in *qk^+/qk^* mutant mice there is reduced...
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FIGURE 9. Overexpression of qkl-5 promotes the expression of Sirt2 mRNA but not SIRT2 protein. A, expression profiles of different qkl variants during differentiation show that qkl-5 mRNA levels were significantly increased by day 3 and qkl-6 mRNA levels were significantly increased by day 6. To further evaluate the role of QKI-5 in regulating Sirt2 expression, CG4-OL cells were transfected with a pIRE2 vector containing a qkl-5 cDNA (see “Materials and Methods”) under differentiation conditions. Transfection with pIRE2-qkl-5 increased expression of qkl-5 mRNA (B). Overexpression of qkl-5 increased the expression of Sirt2 mRNA (C) on days 3 and 6 of differentiation but did not affect the expression of SIRT2 protein (D). RT-PCR (n = 3 biological replicates) and immunoblot (n = 3 biological replicates) data were normalized to β-actin and represented relative to day 0 (mean ± S.D.; two-way ANOVA, *, p < 0.05).

protein expression of SIRT2, whereas the expression of Sirt2 mRNA is not altered in the brain tissue (25). In addition, it has been reported that QKI indirectly controls the expression of SIRT2 during myelination through co-transport with PLP to the myelin sheath (25, 29). Although transport of the SIRT2 protein into the myelin sheath is dependent on PLP (25, 29), the developmental expression of Sirt2 mRNA and protein is most likely regulated by an alternate mechanism(s) because Sirt2 is expressed prior to myelin structural proteins both in vitro (25, 31) and in vivo (25, 28–30). In the present study we have investigated the precise molecular interactions governing the expression of qkl and Sirt2 during OL differentiation.
Our findings reveal that during OL differentiation, there is coordinated expression of qkI and Sirt2 (Figs. 1 and 2) and overexpression of qkI was found to impact the expression of Sirt2 (Figs. 3 and 4) raising the possibility that QKI may directly modulate Sirt2 mRNA expression. In silico analysis revealed two putative quaking response elements, QRE-1 (AUUAAC) at 1639 bp and QRE-2 (ACUAAC) at 1853 bp in the 3' UTR of Sirt2 (Fig. 5A), which conform to the predicted core binding sequence ACUAAY (18) or its variant AUUAAY (8) and binding of QKI with Sirt2 transcripts was confirmed by RNA co-IP (Figs. 5, B and C, and 6A). Binding of QKI to Sirt2 mRNA was observed in both proliferating and differentiating OLs, suggesting that the interaction between QKI and Sirt2 mRNA occurs throughout OL development (Figs. 5, B and C, and 6A). Moreover, we have previously shown expression of Sirt2 drives differentiation at the molecular level by enhancing the expression of MBP (31). Thus, the interaction of QKI with Sirt2 mRNA may be critical early in OL differentiation prior to the onset of myelination. Further analysis revealed that the QRE sequence ACUAAC at 1853–1858 bp was essential for binding of QKI to Sirt2 mRNA (Fig. 7, B and C). This reflected the predicted sequence for high affinity binding (18) and was highly conserved across mammalian Sirt2 transcripts (Fig. 5A). The ability of QKI to bind Sirt2 mRNA indicates that QKI controls SIRT2 protein expression during OL development through an interaction with Sirt2 transcripts.

A reduction in myelin-specific gene transcripts in qk+/qk mice has been shown to be due to post-transcriptional regula-
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Unraveling the molecular interactions between *qkl* and *Sirt2* begins to shed some light on this complexity. Thus, it can be speculated that early in OL development the nuclear isoform *qkI-5* would increase the stability of *Sirt2* transcripts, limiting their availability for translation (Fig. 11). This would delay transition of OPCs to mature OLs thus allowing OPCs to proliferate and migrate from VZ/SVZ to the rest of the CNS. As the OLs reach their final destination they begin differentiating into mature, myelinating OLs where SIRT2 expression in OLs promotes downstream expression of myelin proteins (31). The phenotypes associated with the loss of *qkl-5* in the *qk<sup>v</sup>/qk<sup>v</sup>* (13) and *qk<sup>v</sup>/qk<sup>v-1</sup>* mutants (56) suggests that ability of OLs to sequester mRNAs and limit their availability for translation at early stages is a critical step for proper myelination. In differentiating OLs, cytoplasmic QKI isoforms (e.g. QKI-6) would rapidly increase quantity of *Sirt2* transcripts available for translation. The competition between the differentially localized QKI isoforms would control the availability of target mRNAs for translation and allow OLs to synchronize the timing of myelin protein expression during proliferation, migration, and differentiation. Our findings on the interaction between two key regulators, *qkl* and *Sirt2*, during oligodendroglial differentiation sheds further light into this vital aspect of OL development and myelination.

Materials and Methods

**CG4-OL Cell Culture**—CG4-OL is a O-2A progenitor cell line derived from rat brain, cultured and maintained as previ-
ously described (31–34). Briefly, the CG4-OL cells were cultured on poly-D-lysine (Sigma)-coated tissue culture dishes and maintained as undifferentiated progenitors in GM containing Dulbecco’s modified Eagle’s medium (DMEM), 50 μg/ml of transferrin, 5 μg/ml of insulin, 9.8 ng/ml of biotin, 50 ng/ml of selenium, 1% antibiotic antmycotic solution, and 30% B104 conditioned medium (31, 34). Differentiation of CG4-OL cells was induced by growth in DM containing 2% fetal bovine serum instead of B104-conditioned medium (31, 34). Transfection was carried out using Lipofectamine 2000 (Invitrogen) or polyethylenimine (Polysciences) in serum-free/antibiotic-free media. For GM conditions, cells were harvested 48 h after transfection for RNA and protein extraction. For DM, pcDNA (i.e. qkI) and pIRES2 (i.e. qkI-5) transfections were performed on (day) d0, 2, 4, and d0 and 3, respectively, to maintain consistent levels of expression throughout the 6-day experimental timeline.

Primary Oligodendrocyte Cell Culture and Electroporation—Primary OLs were prepared from C57BL/6 mice at postnatal day 1, as previously described (35, 36). Briefly, mixed glial cells were prepared from the meninges-free cortices isolated from the neonatal pups. The dissected cortices were digested with Accumax solution (Sigma) and passed through a 70-μm nylon cell strainer. The filtered cell suspensions were plated on a poly-D-lysine (Sigma)-coated T75 tissue culture flask and maintained as mixed glial cells in mixed cell medium containing DMEM/F-12, 10% FBS, and penicillin-streptomycin solution. Half of the culture medium was changed every other day. After 7 days, to enrich OLs in the mixed glial cells, mixed cell medium was replaced with OL growth medium (OGM) containing 10 ng/ml of biotin, 5 μg/ml of insulin, 50 μg/ml of transferrin, 2 mM glutamine, 30 nM sodium selenite, 0.1% BSA, 10 nM hydrocortisone, 1% penicillin-streptomycin solution in DMEM/F-12 and 30% of B104-conditioned medium. After 14 days, primary OLs were shaken off (at 200 rpm; 37 °C) from the mixed glial cultures and plated in the OGM supplemented with 10 ng/ml of human recombinant platelet-derived growth factor (PDGF-AA) and 10 ng/ml of basic fibroblast growth factor (bFGF). To induce differentiation, purified primary OLs were plated in OL differentiation medium containing 10 ng/ml of biotin, 5 μg/ml of insulin, 50 μg/ml of transferrin, 2 mM glutamine, 30 mM sodium selenite, 0.1% BSA, 10 mM hydrocortisone, 1% penicillin-streptomycin solution in DMEM/F-12, 5 μg/ml of N-acetyl-L-cysteine and 1% FBS. Transfections were carried out by electroporation (Amaxa nucleofection apparatus, Lonza) in 3–5 × 10⁶ primary OLs using Ingenio electroporation solution (Mirus).
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Bio LLC). Primary OLs were resuspended in OGM (supplemented with PDGF-AA + bFGF) or OL differentiation medium and harvested 6 days after electroporation for protein and RNA extraction.

Vector Construct, Site-directed Mutagenesis—The common coding region of qkl (908 bp) spanning from 489 (exon 1) to 1417 bp (exon 6) containing the RNA binding and dimerization domains (QUA1-KH-QUA2) (12, 15) was amplified by PCR using the forward 5′-AAAAAGATCCAGTTGCTAGTTATGAAGTCCATAGCCGGC-3′ and reverse 5′-GGCAATGGAATAACTCATGCAGCTACCTACGCCC-GC-3′; and Sirt2 3′ UTR-Mut2, 5′-GGCCGGAATCCATCGGTATGCTGTGTAC-3′ and reverse 5′-GGGGATCCGATATGACGTCGTGGAGTCGGGGAAATG-3′. Full-length qkl-5 sequence was amplified by PCR using the forward 5′-GATCCATGGCAGGGGAATGGGAAATGCAGGC-3′ and reverse 5′-GAATTCATAGTTAGTGCCCGGGG-3′ primers and cloned into pGL3-promoter luciferase vector (Promega) at the XbaI site. The two putative QREs in the 3′ UTR were mutated using a QuickChange Lightning kit (Agilent Technologies) with primers, Sirt2 3′ UTR-Mut1, 5′-CCTGACATGATTGTTGCTTTGT-3′ and reverse 5′-ATTGGATACATGGAAGGGC-3′; or Sirt2 3′ UTR-Mut2, 5′-GGACAGGCAACACTAGCCACC-3′ and reverse 5′-TGTTCTCTTTCTCTTTGGTCT-3′; or Sirt2, 5′-AGCAAGGGCAGCACTAGCCACC-3′ and reverse 5′-TTGCCGATATGGATGACC-3′.

Luciferase Reporter Assay—CG4-OL cells maintained in GM were seeded in a white opaque 96-well plate (Costar) and were co-transfected with pcDNA vector or pcDNA-qkl expression vector along with Sirt2 3′ UTR luciferase vector (pGL3-Sirt2 3′ UTR) or putative QREs mutated Sirt2 3′ UTR luciferase vector (pGL3-Sirt2 3′ UTR-Mut1 or pGL3-Sirt2 3′ UTR-Mut2 or pGL3-Sirt2 3′ UTR-Mut1*Mut2). pRL-CMV Renilla luciferase vector was used as a control for normalizing transfection. The Dual-Glo Luciferase Assay System (Promega) was used to measure luciferase activity of cells co-transfected with qkl overexpression vector and wild-type or mutated Sirt2 3′ UTR luciferase vectors. Luciferase assay was performed according to the manufacturer's instruction. Briefly, 48 h after transfection, an equal volume of Dual-Glo reagent was added to the cells in culture medium to measure the firefly lucinescence. Subsequently, Dual-Glo Stop & Glo Reagent was added to inactivate firefly luciferase activity and the Renilla luciferase activity was measured. Relative luciferase activity was calculated by normalizing the firefly luciferase values to its respective Renilla luciferase values. Data were normalized to the Sirt2 3′ UTR co-transfected with pcDNA control vector and represented as percentage luciferase activity.

RNA Isolation and Quantitative Real Time-PCR (qRT-PCR)—Total mRNA from the CG4-OL cells was isolated using RNeasy Mini Kit (Qiagen) or Ambion total RNA miniprep kit (Invitrogen) as per the manufacturer's protocol. First strand cDNA synthesis (reverse transcription) was performed with 500 ng to 1 μg of total RNA using the Quantitect Reverse Transcription Kit (Qiagen) or High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random primers. RT-PCR was carried out according to Ji et al. (31). The following primer pairs were used: (i) qkl-5, forward 5′-CCCTTGCTTTTCTTTGAG-3′ and reverse 5′-CAGGGCAGTCATGGGATCA-3′; (ii) qkl-6, forward 5′-CCTTGCTTTTCTTTGAG-3′ and reverse 5′-GCCCTATGGCTAAATCAATCGACCCTACCC-3′; (iii) qkl-7, forward 5′-CCTTGGCTTTTCTTTGAG-3′ and reverse 5′-AAAAACAGTGGGATGCCACA-3′; (iv) Plp, forward 5′-TGCTGGCTGTGGCTTGAC-3′ and reverse 5′-CTCTACGATGGGATCCCT-3′; (v) Sirt2, forward 5′-AGCAAGGGCAACACTAGCCACC-3′ and reverse 5′-TTGCCGATATGGATGACC-3′.

qRT-PCR was carried out according to Doucette et al., (37), using SYBR select master mix (Applied Biosystems) in a StepOne Real Time PCR System (Applied Biosystems) with gene-specific primers. For analyzing the total Sirt2 mRNA levels, primers were the same as above and for total qkl mRNA levels, forward primer, 5′-TGAATGGGCACTACAGAGAC-3′ and reverse primer, 5′-CAGAAGGACATTATGGTAGGGC-3′, were used. For quantifying the levels of Sirt2 variants, common reverse primer, 5′-GGGAGGCGGAAGTCAGGGAT-3′, was used in combination with variant specific forward primer, Sirt2.1, 5′-CAGGGACCTGATGGGATCCCT-3′, Sirt2.2, 5′-CCAGAGTTGCTACCTGAGCA-3′, Sirt2.3, 5′-CTGCTCCCAGTCCAGGTC-3′. The expression levels of β-actin (primers as indicated above) and 18S ribosomal RNA (18S rRNA) (forward 5′-GCCGGTCTTATTTGTGTGT-3′ and reverse 5′-AGTCGGCATGCTGTGGTCT-3′) were used as endogenous control. The relative levels of transcripts were determined by threshold cycle differences (2^{-ΔΔCt}) of target normalized to the endogenous control as previously described (37).

RNA Co-immunoprecipitation—Immunoprecipitation of mRNA-protein complexes was performed according to Peritz et al., (38). The primary OLs and CG4-OLs were harvested, either in GM or after DM day 6, and lysed with polysome lysis buffer. The mixture was precleared with protein A-agarose beads before immunoprecipitation to remove nonspecific binding to the beads. Subsequently, the supernatant was incubated with 10 μg of anti-QKI antibody or normal rabbit IgG overnight at 4 °C. The following day, fresh protein A-agarose beads were added and incubated for 4 h at 4 °C. Beads were collected after and washed four times with polysome lysis buffer and finally with polysome lysis buffer containing 1 M urea. Next, 0.1% SDS and 30 μg of proteinase K were added to the beads and heated at 50 °C for 30 min to retrieve the protein-RNA complex. RNA isolation and qRT-PCR was performed, as described above. HEK293 cells were co-transfected with wild-type or mutated putative QREs of the Sirt2 3′ UTR along with pcDNA or pcDNA-qkl, to access the critical QRE site(s) involved in QKI
Sirt2 interaction. HEK293 cells were harvested in polysome lysis buffer 48 h after transfection. Lysates were immunoprecipitated with anti-His tag antibody or normal mouse IgG following the protocol described above (38).

**mRNA Stability Assay—**CG4-OL cells maintained in GM were transfected with pcDNA vector or pcDNA-qkI expression vector and the stability of Sirt2 mRNA variants were examined by treating the cells with actinomycin D (Sigma) (20–22). After 48 h of transfection, actinomycin D (10 μg/ml) was added to cells and total RNA was extracted at the indicated time points (0, 15, 30, 60, and 120 min). RNA isolation and qRT-PCR were performed, as described above. Half-life (t_{1/2}) of Sirt2 mRNA variants were calculated as the time required for each mRNA variant to reduce to 50% after actinomycin D treatment from its initial abundance at time 0 min. Half-lives (t_{1/2}) were determined by nonlinear regression analysis (39, 40).

**Western Blotting Analysis—**Total proteins were extracted from CG4-OL cells using RIPA lysis buffer (150 mM NaCl, 0.5% SDS, 1% Triton X-100, 0.1% deoxycholate, 10 mM Tris-HCl, pH 7.2, 5 mM EDTA) at 4 °C. Protein quantification, electrophoresis, and subsequent protein detection was performed as described previously (31). Briefly, proteins (20–40 μg) were separated on 12% SDS-PAGE and transferred to a PVDF membrane. Immunoblots were performed with the following primary antibodies: anti-QKI (1:1000, Abcam), anti-QKI (1:1000, Proteintech Group), anti-Sirt2 (1:3000, Sigma), anti-PLP (1:500, Santa Cruz), anti-MBP (1:1000, Sternberger Monoclonals), anti-β-Actin (1:1000, Santa Cruz), anti-GAPDH (1:10,000, Sigma), and anti-β-tubulin (1:1000, Developmental Studies Hybridoma Bank). Secondary antibodies utilized were goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:3000, Bio-Rad), goat anti-mouse IgG-HRP conjugate (1:3000, Bio-Rad), and goat anti-mouse IgM-HRP conjugate (1:3000, Santa Cruz). Bands were visualized with Clarity™ Western ECL Substrate (Bio-Rad). Densitometric analyses were carried out using Alpha View Imaging software.

**Immunocytochemistry—**Immunocytochemistry was carried out as previously described in Wang et al. (34). Briefly, cells were fixed to cover slips using 4% paraformaldehyde solution and blocked with 3% skim milk in phosphate-buffered saline containing 0.1% Triton X-100, and then incubated with the primary antibody for 1–2 h followed by incubation for 1 h with secondary antibody at room temperature. Cover slips were mounted with Prolong Gold antifade reagent containing DAPI (Invitrogen) to visualize nuclei. The following primary antibodies were used: A2B5 (1:20, ATCC), anti-galactocerebroside (GalC) (1:25, hybridoma) (41), and anti-GFP (1:400, Abcam). The secondary antibodies utilized include Alexa Fluor® 488 goat anti-rabbit IgG (1:200, Molecular Probes) and Alexa Fluor® 594 goat anti-mouse IgG (1:400, Molecular Probes). Each treatment group was assigned an alphanumeric code for blinded cell counts (34). For each cell phenotype (A2B5+ and GalC+), data were collected from >1500 cells and expressed as a percentage of total cells (DAPI+). Cell counts were done using the software program Image Pro® Plus 6.2 (Olympus Canada Inc., Markham, ON).

**Statistical Analysis—**All data are presented as mean ± S.E. Statistically analyses were performed using Student’s t test or ANOVA with Bonferroni’s multiple comparisons post-test (Prism® Software Corporation) as indicated in the figure legends.

**Author Contributions—**A. J. N. and J. R. D. conceived the design and coordinated the study. A. J. N. proofed and revised the manuscript for critical content and interpretation of data and approved the final version for submission. M. P. T. designed, performed experiments, and analyzed the data shown in Figs. 1–8 and together with K. L. F. drafted the article. J. K. G. designed, performed the experiment, and analyzed the data shown in Figs. 9 and 10. K. L. F. and L. S. performed and analyzed the data shown in Fig. 10. S. J. designed and assisted with the analyses of the data in Figs. 9 and 10.

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