**Article**

**Sirt2 is a novel *in vivo* downstream target of Nkx2.2 and enhances oligodendroglial cell differentiation**

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Although Sirt2 is primarily expressed in oligodendrocytes of the central nervous system, its role in oligodendroglial lineage differentiation is not fully understood. Our findings demonstrate that the transcription factor Nkx2.2 binds to the Sirt2 promoter via histone deacetylase 1 (HDAC-1), the binding site for Nkx2.2 maps close to the start codon of the Sirt2 gene, and Nkx2.2 negatively regulates Sirt2 expression in CG4 cells, an oligodendroglial precursor cell line. HDAC-1 knock-down not only significantly attenuates the binding capacity of Nkx2.2 to the Sirt2 promoter but also releases repression of Sirt2 expression by Nkx2.2. Nkx2.2 over-expression down-regulates Sirt2 expression and delays differentiation of CG4 cells; in contrast, up-regulation of Sirt2 does not impact Nkx2.2 expression level. Sirt2 knock-down via RNAi or inhibition of Sirt2 by sirtinol, a Sirt2 activity inhibitor, blocks CG4 cell differentiation. Over-expression of Sirt2 facilitates CG4 cell differentiation at both molecular and cellular levels, enhancing expression of myelin basic protein and facilitating the growth of cell processes. We have conclusively demonstrated that Sirt2 enhances CG4 oligodendroglial differentiation and report a novel mechanism through which Nkx2.2 represses CG4 oligodendroglial differentiation via Sirt2.

**Keywords:** HDAC-1, myelination, Nkx2.2, oligodendrocyte, Sirt2

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**Introduction**

The central nervous system (CNS) consists of neurons and glial cells. Among the glial cells, oligodendrocytes (OLs) provide the myelin sheath that insulates axons of neurons. OLs play an important role in the maintenance of normal CNS function, with abnormal OL function implicated in diseases including multiple sclerosis, transverse myelitis, and optic neuritis (Banik and Pham-Dinh, 2001; de Castro and Bribián, 2005; Rosenberg et al., 2007). Differentiation of OPCs to OLs involves both intracellular and extracellular signals. Recently, Sirt2 has emerged as a regulator of the differentiation process (Li et al., 2007) and is preferentially expressed in oligodendroglial lineage cells in the CNS (Dugas et al., 2006).

Sirt2 is an NAD$^+$-dependent deacetylase belonging to the sirtuin family that includes at least seven members (Sir1–7) in humans (Frye, 2000). Multiple protein targets are identified as sirtuin substrates, including histone and α-tubulin (Vaquiero et al., 2006; Li et al., 2007). Sirtuin family members are class III histone deacetylases, which are important in the epigenetic regulation of diverse cellular processes (Mohn and Schübeler, 2009; Harting and Knöll, 2010) and in nervous system development (MacDonald and Roskams, 2009). Recent studies show Sir-2 (human Sir1 homolog) regulates cell differentiation and calorie restriction in *C. elegans*, whereby over-expression of Sir-2 increases longevity; Sir-2 mutants have a decreased life span (Lamming et al., 2005; Berdichevsky et al., 2006). Sirt1 is also involved in myeloid differentiation and cell cycle regulation (Inoue et al., 2007; Skokowa et al., 2009). However, Sirt2 appears to be involved in the development and differentiation of oligodendroglial lineage cells. Functional genomic analysis reveals that Sirt2 is primarily expressed in OLs of the CNS (Dugas et al., 2006). Sirt2 expression begins only after birth, where it deacetylates α-tubulin in the brain of adult rats (Li et al., 2007). After translation in OLs, Sirt2 is transported to the sheath and associates with myelin. Lack of Sirt2 in the sheath impairs axonal function and leads to progressive axon degeneration (Werner et al., 2007). Several factors affect the developmental process of the neural system, including extracellular signaling, expression of transcription factors and regulation by small RNA (Huang et al., 2010). During early embryonic development, a number of transcription factors and signaling molecules are involved in the emergence and differentiation of oligodendroglial lineage cells (Nicolay et al., 2007). Homeodomain transcription factor Nkx2.2 is a crucial regulator in the developing spinal cord where it may be involved in regulating emergence of OPCs and,
in cooperation with OL lineage gene 2 (Olig2), in induction of ectopic OL differentiation (Guillemot, 2007). An endogenous antisense transcript to Nkx2.2 gene specifies cell fate by directing differentiation of neural stem cells into the oligodendroglial lineage, but simultaneously also induces a modest increase in Nkx2.2 mRNA level (Tochitani and Hayashizaki, 2008). Expression of Nkx2.2 either remains at low levels in mature OLs or represses differentiation and impacts maturation of the progenitor cells into OLs (Wei et al., 2005; Kuhlmann et al., 2008). Histone deacytelyase 1 (HDAC-1) participates in cellular development and differentiation by forming multiprotein complexes (Brunmeir et al., 2009), and plays a crucial role in regulating neurogenesis (Cunliffe, 2004) and OL specification (Cunliffe and Casaccia-Bonnefil, 2006). Loss of its function prevents neural progenitors from specifying into oligodendroglial lineage cells (Cunliffe and Casaccia-Bonnefil, 2006). In addition, HDAC-1 forms a complex with Nkx2.2 in the repression of myelin basic protein (MBP) expression (Wei et al., 2005). Better understanding of how Nkx2.2 regulates OPC differentiation requires knowledge of the mechanism(s) by which Nkx2.2 regulates downstream target gene expression or interacts with proteins important in this process. These downstream molecules likely play key regulatory roles in the differentiation process; however, the detailed mechanisms remain to be resolved.

Here, we report Sirt2 as a new in vivo downstream target of Nkx2.2, which binds to the Sirt2 promoter via HDAC-1 and down-regulates Sirt2 expression. In turn, Sirt2 elevates MBP expression and enhances CG4 oligodendroglial differentiation.

**Results**

**CG4 cell differentiation in vitro**

Sirt2 is expressed in the adult mouse brain, but not in the brain of E18 embryos or newborn mice, or in human embryonic kidney (HEK293) cells (Figure 1A). CG4 cells incubated in differentiation medium (DM; see 'Materials and methods') did not show significant change in expression of Sirt2 or Nkx2.2 but did show increased expression of mature OL markers, MBP and proteolipid protein (PLP) (Buchet and Baron-Van Evercooren, 2009), with this expression reaching a plateau on day 5 (Figure 1C). In growth medium (GM; see 'Materials and methods'), MBP, Sirt2, and Nkx2.2 expression in CG4 cells declined when 50 ng/ml platelet derived growth factor-BB (PDGF-BB) (a repressor of CG4 cell differentiation and maturation) was added every day for 6 days (Figure 1B); these cells retained a precursor bipolar morphology, indicating the cells remained undifferentiated. In general, GM keeps the majority of CG4 cells in an undifferentiated state, but some cells will begin to differentiate over time even if being continually fed with GM. When PDGF-BB is added to the GM for the first week even fewer CG4 cells differentiate. Basic fibroblast growth factor (10 ng/ml) had no detectable effect on CG4 cell differentiation (data not shown).

**Characterizing the binding of Nkx2.2 to Sirt2 promoter**

Transcription factors STAT-1, -3, Nkx2.2, and Olig2 were evaluated in CG4 cells for interaction with Sirt2 and MBP promoter regions. STAT-1, -3, and Olig2 bound to neither the Sirt2 nor the MBP promoter. Regardless of the stage of differentiation, Nkx2.2 bound to the Sirt2 promoter but not to the MBP promoter (Figure 2), as determined in a binding assay using lysates from cells cultured in GM, DM, and in GM containing 50 ng/ml PDGF-BB.

The Sirt2 promoter was truncated with biotinylated primers to generate fragments with overlapping base pair sequences. Use of primers covering sequences from -168 to +5 bp showed potential binding sites of Nkx2.2 in sequences from -168 to -98 bp and from -78 to +5 bp (Figure 3A and B). To accurately map out the binding sequence region, Nkx2.2–GST was expressed using the E. coli expression system; however the purified Nkx2.2–GST did not bind to the Sirt2 promoter (Figure 3C). When Nkx2.2–GST was mixed with lysate of CG4 cells, the ability to bind to the Sirt2 promoter (Figure 3D) was recovered, and wild-type Nkx2.2 also reappeared, as indicated by bands on X-ray films exposed for 60 sec (Figure 3D, bottom).

**Figure 1** Sirt2 is expressed in adult mouse brain and in differentiated CG4 cells. (A) Sirt2 expression in adult mouse brain and CG4 cells. Whole brain (adult, newborn or embryo E18) and cell lysates (CG4 or HEK293) were analyzed by western blot and 10 μg protein was loaded for each lane. (B) PDGF impedes CG4 cell differentiation. CG4 cells in GM were treated with PDGF-BB (50 ng/ml) for 6 days. MBP, Sirt2, and Nkx2.2 mRNA and/or protein levels were evaluated. MBP expression is visible (with a longer exposure time) although weak, and MBP, Sirt2, and Nkx2.2 expression gradually decline over the treatment period. (C) MBP expression increases during differentiation, whereas Nkx2.2 levels do not change. Sirt2 mRNA (top panel) remains unchanged, although its protein expression appears to peak at day 3 (bottom panel).
Figure 2 Binding of Nkx2.2 to the Sirt2 promoter in CG4 cells. (A) Association between Nkx2.2 and the Sirt2 promoter was determined by DNA–protein pull-down assay. Olig2, STAT-1, and -3 did not bind to the Sirt2 or MBP promoter. Nkx2.2 binds to the Sirt2 promoter but not to the MBP promoter. (B) Binding of Nkx2.2 to the Sirt2 promoter is not affected by GM, DM, or treatment with PDGF.

Figure 3 Binding between the Nkx2.2 protein and the Sirt2 promoter is dependent on HDAC-1. The two regions of DNA where Nkx2.2 binds to the Sirt2 promoter were shortened to −168 to −98 bp and −78 to +5 bp. (A) The Sirt2 promoter was divided into three parts for the binding assay. Lanes A–H: described on upper bars, respectively; lane FL: full-length of Sirt2 promoter; Beads: streptavidin-conjugated agarose beads control. (B) DNA-covering fragment F–H (Sirt2 promoter) was recovered from anti-Nkx2.2 antibody precipitated DNA–protein complex, and Nkx2.2 was confirmed to bind to the recovered Sirt2 promoter by ChIP (lane Sirt2). (C) Streptavidin pull-down assay was carried out with E. coli lysate containing Nkx2.2-GST protein without mammalian cofactor(s). Fragments D–H derived from the Sirt2 promoter were used. None of the Sirt2 promoter fragments (D–H) were capable of binding to Nkx2.2-GST. (D) The pull-down assay was carried out with a mixture of E. coli lysate and CG4 lysate. The top panel film was exposed for 5 sec; the area in broken lined frame was exposed for 60 sec and shown in the lower panel. DNA fragments E, F, and H bound to Nkx2.2, suggesting a cofactor in CG4 lysate mediated the interaction. (E) Co-immunoprecipitation was performed with anti-Nkx2.2 or anti-HDAC-1 antibody in CG4 cell lysates. Nkx2.2 is present in the immuno-complex precipitated by anti-Nkx2.2 antibody (left panel) and HDAC-1 is present in the immuno-complex precipitated by anti-HDAC-1 antibody (right panel). (F) Three double-stranded fragments of siRNA against human HDAC-1 were synthesized and transfected into SH-SY5Y cells and siRNA-1 is most efficient in down-regulating HDAC-1 expression (lane 1). (G) ChIP-PCR with anti-Nkx2.2 antibody was performed after HDAC-1 knock-down or over-expression. The binding capacity of Nkx2.2 to the Sirt2 promoter decreased when HDAC-1 expression was knocked-down (lane 2, top panel). Paradoxically, over-expression of HDAC-1 did not increase the binding capacity of Nkx2.2 to Sirt2 promoter (lane 5, top panel). In contrast, the expression level of Sirt2 increased when HDAC-1 was knocked-down (lane 2, middle panel) and Sirt2 expression level decreased when HDAC-1 was over-expressed (lane 5, middle panel). CG4: CG4 cell lysate as control.
These results suggest Nkx2.2 binding to the Sirt2 promoter might be indirect and requires cofactors.

**HDAC-1 as a cofactor of the binding of Nkx2.2 to the Sirt2 promoter**

Nkx2.2 was reported to form a complex with mSin3A or HDAC-1 during OL differentiation (Wei et al., 2005). Using the transcription element search system program, we identified Evx-1 and GATA-2 as additional potential cofactors since they have binding sites close to the Nkx2.2-binding region. Co-immunoprecipitation assays indicated that Evx-1, GATA-2, and mSin3A do not interact with Nkx2.2 in CG4 cells (data not shown). However, Nkx2.2 does form a complex precipitated by HDAC-1 antibody and HDAC-1 appeared in a complex precipitated by Nkx2.2 antibody (Figure 3E, lane 1), thus suggesting Nkx2.2 forms a complex with HDAC-1 in CG4 cells.

To further explore the role of HDAC-1 in mediating the binding of Nkx2.2 to Sirt2 promoter, we over-expressed or siRNA knocked-down HDAC-1. Three HDAC-1 specific fragments of siRNA were designed to partially knock-down HDAC-1 expression; HDAC-1 siRNA-1 had the most prominent effect (Figure 3F). As shown in Figure 3G, HDAC-1 knock-down significantly attenuated the binding capacity of Nkx2.2 to Sirt2 promoter (lane 2, top panel) but the binding capacity was not significantly affected by HDAC-1 over-expression (lane 5, top panel); Sirt2 mRNA was significantly increased when HDAC-1 expression was down-regulated by siRNA (lane 2, middle panel), but was significantly decreased by HDAC-1 over-expression (lane 5, middle panel). These data show HDAC-1 likely functions as an auxiliary factor in a complex with Nkx2.2 binding to the Sirt2 promoter.

**Impact of over-expression of Sirt2 on CG4 cell differentiation**

Western blot analysis revealed that Nkx2.2 over-expression negatively regulates Sirt2 expression in CG4 cells (Figure 4A), while Sirt2 over-expression did not alter the Nkx2.2 expression level (Figure 4B). Sirt2 over-expression did facilitate the differentiation of CG4 cells into OLs in DM, with MBP expression appearing earlier (on day 2 after transfection) (Figure 4D, top panel) than controls (Figure 4C, bottom panel). As shown in Figure 4D, in DM the MBP expression level in Sirt2 over-expressing CG4 cells decreased after day 3, plateauing by day 4 (top panel); in GM, Sirt2 over-expression induced MBP expression from day 5 onwards (bottom panel), but it was still lower than in differentiated CG4 cells (bottom panel, lane DC). In contrast, Nkx2.2 played a suppressive role on CG4 cell differentiation in DM where MBP expression was significantly inhibited and only appeared after day 5 (Figure 4E).

**Impact of down-regulating Sirt2 expression on CG4 cell differentiation**

We further examined the effect of suppressing Sirt2 activity on the differentiation of these cells. In CG4 cells cultured in DM with 25 μM sirtinol, a specific inhibitor of Sirt2 activity, MBP expression was significantly suppressed and was almost undetectable (Figure 5A) compared with control (Figure 4C). RNA interference was also used to knock-down Sirt2 expression. We synthesized two fragments of double-stranded siRNA of Sirt2 (Li et al., 2007) matching rat mRNA; both fragments (Sirt2 siRNA-1 and -2) significantly reduced the Sirt2 mRNA level (Figure 5B).

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**Figure 4** Regulatory relationship between Nkx2.2 and Sirt2 and effects on CG4 cell differentiation. (A and B) Nkx2.2 and Sirt2 were cloned into pcDNA3.1/myc-His, respectively, after transfection, cells were harvested and analyzed for protein expression at 36 and 60 h. (C) Wild-type CG4 cells (top) or cells transfected with blank vector (bottom) were plated in DM, and MBP expression detected with western blot. (D) CG4 cells were transfected with Sirt2-pcDNA and plated in DM (top panel) or GM (bottom panel). DC: differentiated cells as positive control. (E) CG4 cells were transfected with Nkx2.2-pcDNA and plated in DM.
CG4 cells in DM were transfected for a second time with Sirt2 siRNA-2 72 h after first Sirt2 siRNA-2 transfection. MBP expression was significantly reduced in the Sirt2 siRNA-2 transfected cells compared with control siRNA, and was almost undetectable even on day 6 (Figure 5C, bottom panel).

**Impact of Sirt2 expression on CG4 cell morphology**

Differentiation of oligodendroglial lineage cells is a very complicated process, involving the expression of myelin proteins, such as MBP, and major changes in cell morphology. Thus, we examined the effect of Sirt2 over-expression on CG4 cell morphology. Undifferentiated wild-type CG4 cells had a bi- or tri-polar morphology (Figure 6A), whereas differentiated CG4 cells had numerous extended, web-like cytoplasmic processes (Figure 6B). Undifferentiated CG4 cells transfected with Nkx2.2 and blank vectors largely retained an undifferentiated morphology (Figure 6D and F), whereas undifferentiated CG4 cells transfected with Sirt2 possessed more cytoplasmic processes (Figure 6G and H, arrows).

**Discussion**

CG4 cells are a cell line of OPCs where MBP protein expression should be minimal. MBP protein levels are very low in intact CG4 cells, being only visible after a long exposure time; mRNA levels, however, are easily detected after RT-PCR amplification. Expression of MBP protein and PLP mRNA (markers of OL differentiation) significantly increase when CG4 cells are cultured in DM and, hence, the CG4 cell line is a useful tool for investigating OL differentiation and myelination. In our study, MBP and Sirt2 protein and mRNA expression remained at low levels in undifferentiated CG4 cells, and Sirt2 protein expression begins just as OLs begin to differentiate, indicating that Sirt2 protein may facilitate CG4 cell differentiation into myelin-producing OLs.

We also identified an association between the Nkx2.2 protein and the Sirt2 promoter but did not observe binding of Nkx2.2 protein to the MBP promoter, contradicting a previous report (Wei et al., 2005). We found binding of Nkx2.2 protein to the Sirt2 promoter was not influenced by DM and PDGF treatment,
which induced and suppressed the differentiation of CG4 cells, respectively. In the CG4 cell line, binding of Nkx2.2 protein to the Sirt2 promoter seems ubiquitous, being not influenced by the developmental state, although the biological function of such binding is very likely affected. A consensus Nkx2.2-binding sequence has previously been identified (Watada et al., 2000); however, we did not find the corresponding sequence in the Sirt2 promoter, hence Nkx2.2 binding to the Sirt2 promoter may be indirect, requiring at least one cofactor. Co-immunoprecipitation results showed that Nkx2.2 and HDAC-1 can form a complex. HDAC-1/-2 has been implicated in regulating embryonic development and cell differentiation (Brunmeir et al., 2009) and may function as a cofactor to control cell development and differentiation.

Our results further indicate Nkx2.2 represses Sirt2 transcription/expression in an HDAC-1 dependent manner, although overexpression of HDAC-1 did not increase the physical binding of Nkx2.2 protein to Sirt2 promoter. One possibility is that endogenous HDAC-1 protein in CG4 cells is ubiquitous or abundant enough thus making exogenous HDAC-1 protein unnecessary or excessive. Another possibility is that any increase in binding capacity of Nkx2.2 protein to the Sirt2 promoter is too small to be detected, but it could induce detectable decreases in Sirt2 expression. We showed that Sirt2 over-expression promoted MBP protein expression in differentiated as well as undifferentiated CG4 cells and that the MBP protein expression in Sirt2 over-expressing cells was quickly attenuated, with levels plateauing earlier compared with control differentiated CG4 cells. Thus, expression of Sirt2 protein by itself is not sufficient for stably driving CG4 cells into a differentiation mode; other cofactors are necessary. In contrast, Nkx2.2 gene over-expression suppressed MBP protein expression and CG4 cell differentiation, supporting previous finding that Nkx2.2 represses differentiation of OLs (Wei et al., 2005). These observations collectively support a role for Sirt2 in enhancing MBP protein expression and facilitating CG4 cell differentiation.

Inhibition of Sirt2 using either sirtinol or siRNA reduced expression of MBP protein. Sirtinol also completely suppressed differentiation of CG4 cells while the Sirt2 siRNA-transfected CG4 cells appeared to at least begin to differentiate. Suppression of Sirt2 expression by siRNA may be less efficient than sirtinol in terms of affecting Sirt2’s biological function, perhaps due to lower transfection efficiency, but the findings support a positive role for Sirt2 in the differentiation of CG4 cells.

A previous study (Li et al., 2007) using primary cell cultures reported that Sirt2 protein expression marked the entry of OPCs into the immature stage of OL differentiation and suppressed MBP protein expression and the maturation of OPCs into OLs. Our findings from over-expression, knock-down expression, and use of sirtinol consistently showed that Sirt2 protein enhances MBP expression and facilitates CG4 cell differentiation. The reasons behind these discrepancies are not clear. However, the data from Li et al. (2007) and the findings from our study both show that Sirt2 protein expression profile is consistent with that of MBP expression, with a gradual increase after birth and levels peaking in the adult animal. Hence, the notion that Sirt2 represses MBP expression (Li et al., 2007) is at odds with what is observed or would be expected, as the myelination process increases soon after birth at a time when Sirt2 protein expression is also increasing. Furthermore, we detected all four MBP isoforms (17.2, 18.5, 20.2, and 21.5 kDa) for the MBP protein expression profile. In contrast, Li et al. (2007) included only a single band in western blots and did not identify the MBP isoform; hence, the antibody used in their study may have led them to a different interpretation.

We further observed morphologically that Sirt2 over-expression facilitated CG4 cell differentiation with the generation of more cytoplasmic processes. Sirt2 transfection was purposely carried out at low cell density with low transfection efficiency so only a few cells were present in each field of view. Due to the strong correlation between morphology and biological function, Sirt2 appears to play an important role in driving the cells to extend more cytoplasmic processes in the early stages of OPC differentiation. This result is consistent with an earlier increase in MBP protein expression, which we observed following Sirt2 gene over-expression in GM after 5 days. Overall, our results indicate Sirt2 facilitates CG4 oligodendroglial cell differentiation and that Nkx2.2 via HDAC-1 inhibits this process by binding to a specific region on the Sirt2 promoter (Figure 7). The precise mechanism by which Sirt2 facilitates CG4 oligodendroglial cell differentiation requires further investigation.

**Materials and methods**

**B104-conditioned medium preparation**

B104 neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco) containing 10% fetal bovine serum to 80% confluence, washed with Puck’s solution (0.1% glucose, 0.8% NaCl, 0.04% KCl, 0.006% KH2PO4, 0.0048% Na2HPO4, and 0.002% phenol red) three times, and then transferred to DMEM/F12 with 1 mg/ml transferrin, 100 μM putrescine dihydrochloride, 3 mM sodium selenite, and 2 μM progesterone. After 2–3 days, the culture medium was harvested and 1 μg/ml phenylmethylsulfonyl fluoride (PMSF) added. The medium was centrifuged at 1000 g, 4°C for 10 min, and the supernatant was filtered (0.22 μm) and stored at −20°C.

**Cell culture, treatment and transfection**

CG4 cells were grown, passaged, and harvested as described in Louis et al. (1992). To induce cell differentiation, cells were plated...
in DM consisting of DMEM containing 50 μg/ml transferrin, 9.8 ng/ml biotin, 50 ng/ml sodium selenite, 5 μg/ml insulin, 2% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The medium was renewed every other day for 6 days. (i) Cells treated with PDGF were plated in GM (30% B104-conditioned DMEM containing 50 μg/ml transferrin, 9.8 ng/ml biotin, 50 ng/ml sodium selenite, 5 μg/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin) and PDGF-BB (Sigma) added to a final concentration of 50 ng/ml every day for 6 days. (ii) CGa cells were plated in GM or DM and sirtinol (Sigma) was added to a final concentration of 25 μM (dissolved in DMSO). The medium was renewed and sirtinol refreshed every day for 6 days. (iii) Control cultures, treated with only DMSO, were also carried out using identical protocols. Before transfection, CGa cells were plated into 6- or 24-well plates in GM without antibiotics. The transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Control vectors or expression plasmids (1–5 μg) were transfected as control. The medium was switched to GM or DM with antibiotics 12 h after transfection. Three days after the first transfection, the cells were transfected for a second time with the same DNA constructs. The cells were harvested to measure RNA and protein levels every day after the first transfection.

RT-PCR

The primers were designed to amplify mRNA transcript fragments of Sirt2, MBP, PLP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for Sirt2 (rat) were forward 5′-AGCAAGGCACCTAGCCACC-3′ and reverse 5′-TGTTCCCTTTTCACTGTGTC-3′; for Sirt2 (human) were forward 5′-AGCAAGGCACCTAGCCACC-3′ and reverse 5′-GGTTTCTTCCCTCCTCTGTGTC-3′; for MBP were forward 5′-GATGGCTTCTCTCAAGGACACC-3′ and reverse 5′-GAGGATGGAGAATCCAGAGG-3′; for PLP were forward 5′-TGTGCTGTCTGCTGTAC-3′ and reverse 5′-TCTATGGGAGATCAGAATGGT-3′; and for GAPDH were forward 5′-ACCAAGATCTTGCCATTAC-3′ and reverse 5′-TCCACACCCCTGTTGCTGTA-3′. Total RNA was isolated from freshly harvested cells with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription of 1 μg of total RNA was conducted with SuperScript reverse transcriptase II (Invitrogen). PCR amplification was carried out for 21–25 cycles, with each cycle consisting of denaturation for 40 sec at 94°C, annealing for 40 sec at 50°C–60°C, and an extension for 40 sec at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

Plasmid construction

Rat cDNAs of Sirt2, Nkx2.2, and HDAC-1 were amplified by PCR and the products isolated and cloned into T-easy vector for sequencing followed by sub-cloning into expression vectors as described below. Rat Sirt2 cDNA (HindIII and EcoRI fragment) was amplified by PCR with forward primer 5′-AAGCTTCCAGAGATGGACTTCCTAGGC-3′ and reverse primer 5′-GAATTTCTAGTTCTCCCTTCTGG-3′ and cloned into pcDNA3.1/myc-His (A). Expression of a non-fusion protein was expected due to a stop codon in the reverse primer. Sirt2 was cloned into pIRE2-EGFP at NheI and EcoRI sites after PCR using the following primers: forward 5′-GCTAGGGCAAGTGGACTTCCCTAGGC-3′ and reverse 5′-GAATTTCTAGTTCTCCCTTCTGG-3′. Rat Nkx2.2 cDNA was cloned into pcDNA3.1/myc-His (A) and pGEX-6P-1 at BamHI and EcoRI sites, or pIRE2-EGFP at BglII and EcoRI sites by isoacceptors BglII that matches BamHI, with forward primer 5′-GGATCCATTTGTGCTGACCAACAAAAAG-3′ and reverse primer 5′-GAATTTCTACCAAGTCTCAGTGGCC-3′. HDAC-1 was cloned into pcDNA3.1/myc-His (B) at BamHI and Xhol sites, with forward primer 5′-GGATCCATTTGTGCTGACCAACAAAAAG-3′ and reverse primer 5′-CTCGAGCTCTAGGCGATCCATTTCTGACCAACAAAAAG-3′. (ii) Control cultures, treated with only DMSO, were also carried out using identical protocols. Before transfection, CGa cells were plated in GM or DM and sirtinol (Sigma) was added to a final concentration of 25 μM (dissolved in DMSO). The medium was renewed and sirtinol refreshed every day for 6 days. (iii) Control cultures, treated with only DMSO, were also carried out using identical protocols. Before transfection, CGa cells were plated into 6- or 24-well plates in GM without antibiotics. The transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Control vectors or expression plasmids (1–5 μg) were transfected as control. The medium was switched to GM or DM with antibiotics 12 h after transfection. Three days after the first transfection, the cells were transfected for a second time with the same DNA constructs. The cells were harvested to measure RNA and protein levels every day after the first transfection.

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Cloning of MBP and Sirt2 promoter regions

Primers and biotinylated primers were from Invitrogen. For MBP promoter cloning, we used forward primer 5′-GTTTCTGTAAGAAAAGGGACAG-3′ and reverse primer 5′-TTCGGAAAGCTGCTTGCGGATC-3′ (Wei et al., 2003). The rat Sirt2 promoter sequence is unknown and currently not available, hence human Sirt2 promoter was used for Sirt2 promoter cloning, using forward primer 5′-GGTTTCTTCACTGCTTCATGAC-3′ and reverse primer 5′-TGCCATGCGGCGTGCTGAAG-3′ (Voelter-Mahlknecht et al., 2005). Genomic DNA was extracted from cultured CG4 cells or SH-SY5Y cells. In brief, 1 × 10⁶ cells were harvested by centrifugation, then 400 μl of lysis buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 100 μg/ml protease K) added. The mixed sample was vortexed for 10 sec, kept at room temperature for 1 h, and then centrifuged at 12000 g for 1 min. The supernatant was mixed with the same volume of isopropanol, held at room temperature for 2 min, and then centrifuged at 12000 g for 5 min. The pellet was washed with 70% ethanol by centrifugation and dissolved in TE buffer for PCR for 25 cycles of denaturation for 40 sec at 94°C, annealing for 60 sec at 65°C, and an extension for 2 min at 72°C. PCR products were purified and inserted into T-easy vector for sequencing. The recombinant vectors were used for PCR templates in the following probe preparations.

Biotinylated probes

To obtain biotinylated full-length MBP promoter probe, we used forward primer biotin-5′-AAGTTTCCTGTGAGAAAAGGGACAG-3′ and reverse primer biotin-5′-CTCTGGAAAGCTGCTTGCGGATC-3′. For the biotinylated full-length Sirt2 promoter probe, we used forward primer 5′-biotin-GTAGGAGGTGATTTCCCTAGG-3′ and reverse primer 5′-biotin-CAGGGCGCTGTGCTGACCAACAAAG-3′. PCR was carried out for 35 cycles with 40 sec at 94°C, 60 sec at 60°C, and 72°C for 2 min.
Subsequently, the Sirt2 promoter was fragmented into three parts. For the sequence between −893 and −553 bp, we used forward primer 5′-biotin-GGTAGAAATGTGGGTTAG-3′ and reverse primer 5′-biotin-GATCAGCCAGCTTGTTGAAA-3′. For the sequence between −574 and −272 bp, we used forward primer 5′-biotin-CAGGCCGGTCTCCACCTC-3′ and reverse primer 5′-biotin-CCATTGTGTTAAAGGCCCCTC-3′. For the sequence between −294 and +5 bp, we used forward primer 5′-biotin-GGCGGCC TTTAACCAATGG-3′ and reverse primer 5′-biotin-GCCATGGGGCGCG GTGCTGAAG-3′. For these three probes, PCR conditions were 94°C for 40 sec, 60°C for 40 sec, and 72°C for 60 sec. For the sequence between −294 and −148 bp, we used primers 5′-biotin-GGCCG CCTTTACCAATGG-3′ and 5′-biotin-GAACTACAATCTCTAGCAGAC A-3′. For the sequence between −168 and +5 bp, we used primers 5′-biotin-GTCTGCTAGAGTGTGTTACG TTTTACCAATGG-3′ and 5′-biotin-GCCATGGGGCGCGGTGCTGAAG-3′. Sequences were further truncated. For the sequence from −168 to −98 bp, primers were 5′-biotin-GTCTGCT GAGAGTTGTAGTTC-3′ and 5′-biotin-CTCTGCCCCGTGACGGCACCA-3′. Primers for the sequence from −119 to −57 bp were 5′-biotin-TGTGCTCCAGGCCCCAGAG-3′ and 5′-biotin-ACTGTCCTGCCGTAC CGACTG-3′, and for −78 to +5 bp were 5′-biotin-CAGTCTGGTACG GGCACAGT-3′ and 5′-biotin-GCCATGGGGCGCGGTGCTGAAG-3′. PCR conditions for these probes were 94°C for 40 sec, 55°C for 30 sec, and 72°C for 30 sec. After agarose gel electrophoresis, all probes were isolated, purified, and quantified with a DNA gel extraction kit (Invitrogen) according to the manufacturer’s instructions.

**Binding assay**

The same quantity of cultured CG4 cells for each sample was lysed with modified RIPA buffer containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM Na3VO4, 1 mM EDTA, and 0.5% protease inhibitor cocktail (Sigma). The cells were stirred in the RIPA buffer and held on ice for 10 min, then centrifuged at 14000 g, 4°C for 15 min. A binding assay (Deng et al., 2003) was conducted with the following modifications. Biotinylated DNA probe (5 μg) was added to the supernatant and the samples incubated at 4°C for 12 h with gentle agitation. Streptavidin–agarose beads (20 μl) were added to each sample and incubated at 4°C for 1 h. Following incubation, beads were washed and centrifuged four times with 1 ml RIPA buffer at 4°C. Beads were resuspended in 30 μl of 2× SDS loading buffer, boiled for 5 min, and supernatant used for western blot analysis. To identify direct or indirect binding of Nkx2.2 to the Sirt2 promoter, Nkx2.2 was cloned into pGEX-6P-1 and expressed as a fusion protein with glutathione S-transferase (Nkx2.2–GST) in E. coli after induction with 2.0 mM IPTG at 26°C for 2 h. The Nkx2.2–GST protein was used as a cofactor free protein source. The E. coli cells were lysed in PBS (0.8% NaCl, 0.02% KCl, 0.144% Na2HPO4, 0.024% KH2PO4, pH 7.4) with 1% Triton X-100 (v/v), 1 mM PMSF, 1 mM DTT, 0.6% sarkosyl, and 0.5 mg/ml lysozyme with stirring and left on ice for 20 min. The sample was sonicated at 40% maximum power for 2× 20 sec and centrifuged at 14000 g, 4°C for 15 min. The supernatant solutions either alone or combined with CG4 cells lysates were used as the Nkx2.2 source in the binding assay. Equal biotinylated DNA fragments from the Sirt2 promoter (from PCR above) were added to the supernatant solutions and incubated at 4°C overnight. This procedure was also performed for CG4 cells as described above.

**ChIP and PCR**

SH-SY5Y cells were grown in DMEM/F12 supplemented with 10% fetal calf serum and 5% CO2. Approximately 5 × 106 cells were used for the ChIP assay (Upstate) according to the manufacturer’s protocol. Briefly, 37% formaldehyde was directly added to the tissue culture medium to a final concentration of 1% and incubated for 10 min. Cells were washed, scraped, and collected by centrifugation at 2000 g for 2 min. Cells were then resuspended in SDS lysis buffer containing protease inhibitor and kept on ice for 10 min. The isolated chromatin was sheared by sonication to an average size of 200–1000 bp. Samples were centrifuged at 13000 g, 4°C for 10 min and the supernatant retained. Before pre-clearing with protein A agarose, the DNA–protein complex was precipitated by adding anti-Nkx2.2 antibody. The DNA was recovered by phenol–chloroform extraction and used as a template for PCR. The forward and reverse primers used were 5′-GGCGGCCCTTTACCAATGG-3′ and 5′-GCCATGGGGCGCGGTGCTGAAG-3′, respectively. Amplification was performed using EcoTaq polymerase (Lucigen), with thermocycler settings beginning with a hot-start of one cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 40 sec, 55°C for 40 sec, and 72°C for 40 sec. Resultant aliquots were analyzed by agarose gel electrophoresis. Western blot assays and co-immunoprecipitation

Adult and newborn C57 black mice were killed by decapitation following isoflurane inhalation overdose. The brain tissues were homogenized in ice-cold modified RIPA buffer containing protease inhibitor cocktail with a Dounce homogenizer, and cultured CG4 cells were lysed with the same RIPA buffer at 4°C. The lysates were centrifuged at 12000 g, 4°C for 15 min. For co-immunoprecipitation, the supernatant was pre-cleared with protein A agarose beads, quantified for protein concentration and diluted to 1 mg/ml. Aliquots of the specific antibodies or normal IgG (20 μg) were added to the supernatant for each sample. For western blot analysis, the supernatants were mixed with loading buffer and boiled for 5 min. Supernatants were then loaded on 10% or 12% SDS–polyacrylamide gel for electrophoresis. Membranes were blocked with 5% skim milk or 3% BSA (anti-MBP and anti-Sirt2) for 1 h at room temperature and incubated overnight at 4°C (Ji et al., 2006) with the following primary antibodies, respectively: goat anti-MBP, rabbit anti-Olig2, anti-mSin3A, anti-gata2, and anti-HDAC-1, mouse anti-Evx-1 and anti-β-actin antibody (Santa Cruz), rabbit anti-STAT-1 and mouse anti-STAT-3 antibody (Cell-Signaling), mouse anti-Nkx2.2 antibody (Developmental Studies Hybridoma Bank, University of Iowa), or rabbit anti-Sirt2 antibody (Acris antibodies GmbH). Membranes were then washed with PBST (PBS with 0.1% Tween-20) for 3× 10 min on a shaker. Horseradish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibody (Bio-Rad) or HRP-conjugated anti-goat antibody (Santa Cruz) was used as secondary antibody before visualization with western lighting chemiluminescence reagent (PerkinElmer Life Science). Membranes were stripped with buffer (65 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at 60°C for 30 min with gentle agitation and reused for western blot analysis as described above.

**Cell morphology**

Nkx2.2 or Sirt2 cDNA was cloned into a bicistronic vector pIRE52-EGFP (Clontech) and transfected into CG4 cells with a blank vector as a control, then plated in GM. Five days after
transfection, cell morphology was observed with fluorescence microscopy and images recorded at 100× magnification.

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**Conflict of interest**: none declared.

**References**


Sirt2 enhances OL differentiation