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## Neuron-specific specificity protein 4 (Sp4) bigenomically regulates the transcription of all mitochondria- and nucleus-encoded cytochrome c oxidase subunit genes in neurons

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### Abstract

Neurons are highly dependent on oxidative metabolism for their energy supply, and cytochrome c oxidase (COX) is a key energy-generating enzyme in the mitochondria. A unique feature of COX is that it is one of only four proteins in mammalian cells that are bigenomically-regulated. Of its thirteen subunits, three are encoded in the mitochondrial genome and ten are nuclear-encoded on nine different chromosomes. The mechanism of regulating this multisubunit, bigenomic enzyme poses a distinct challenge. In recent years, we found that nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) mediate such bigenomic coordination. The latest candidate is the specificity factor (Sp) family of proteins. In N2a cells, we found that Sp1 regulates all 13 COX subunits. However, we discovered recently that in primary neurons, it is Sp4 and not Sp1, that regulates some of the key glutamatergic receptor subunit genes. The question naturally arises as to the role of Sp4 in regulating COX in primary neurons. The present study utilized multiple approaches, including chromatin immunoprecipitation, promoter mutational analysis, knockdown and over-expression of Sp4, as well as functional assays to document that Sp4 indeed functionally regulate all 13 subunits of COX as well as mitochondrial transcription factors A and B.

### Keywords

Cytochrome c oxidase; depolarization; mitochondria; transcription factor; TTX; Sp4

### Introduction

The brain is a highly energy-demanding organ, and much of the energy utilized by neurons is derived from oxidative metabolism in the mitochondria. Cytochrome c oxidase (COX) plays an important role in this process, as it is one of three proton-pumping, energy-generating enzymes of the inner mitochondrial membrane. It is also the terminal enzyme of the electron transport chain, without which energy metabolism cannot be carried to completion (Wikström et al., 1981; Wong-Riley, 1989).

In neurons, COX activity is controlled by the demands of neuronal activity. A major component of neuronal activity is the repolarization of membrane potential after excitatory depolarization, and it is highly energy-dependent (Wong-Riley, 1989). Energy metabolism and neuronal activity are, therefore, tightly coupled processes (Lowry, 1975, Wong-Riley, 1989). When neuronal activity is increased, so does the demand for ATP from the oxidative pathway, and the level of COX is up-regulated; likewise, reducing neuronal activity leads to

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the down-regulation of COX (Wong-Riley, 1989; Liang et al., 2006). Thus, COX serves as a sensitive and reliable metabolic marker for neuronal activity.

COX is one of only four known proteins in mammalian cells that are bigenomic, i.e., derived from both the nuclear and the mitochondrial genomes. Three of its 13 subunits are mitochondrial-encoded and they form the catalytic core of the enzyme, whereas the other 10 are nucleus-specified (Kadenbach et al., 1983) on nine different chromosomes. The coordinated regulation of this multisubunit, multi-chromosomal, bigenomic enzyme is indeed challenging. In searching for transcription factor candidates, we found that both nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) directly regulate all 10 nucleus-encoded *COX* subunit genes (Ongwijitwat et al., 2006; Dhar et al., 2008). The fact that each of them regulates mitochondrial transcription factors A, B1, and B2 (TFAM, TFB1M and TFB2M, respectively), all of which, in turn, regulate the transcription and replication of mitochondrial DNA (mtDNA) (Virbasius and Scarpulla, 1994; Gleyzer et al., 2005), indicates that NRF-1 and NRF-2 indirectly regulate the 3 mitochondrial-encoded *COX* subunit genes as well. More recently, we found that each of these two transcription factors also regulates key subunit genes of the glutamatergic NMDA receptors (Dhar and Wong-Riley, 2009; Priya et al., 2013a). Thus, NRF-1 and NRF-2 mediate the tight coupling between neuronal synaptic transmission and energy metabolism at the molecular level.

Besides NRF-1 and NRF-2, promoters of a number of nucleus-encoded *COX* subunit genes reportedly also contain putative binding sites for specificity protein 1 (Sp1) (Grossman and Lomax, 1997; Ongwijitwat and Wong-Riley, 2004). Indeed, we recently documented that Sp1 regulates all *COX* subunit genes in neuroblastoma N2a cells (Dhar et al., 2013). However, when we started analyzing Sp factors' role in regulating mediators of synaptic transmission in primary neurons, we found that it is Sp4, and not Sp1, that regulates the expression of NMDA receptor subunit genes (Priya et al., 2013b). We also found that the nuclear level of Sp4 is much higher in primary neurons than in N2a cells, whereas the converse is true for Sp1 (Priya et al., 2013b). These findings compelled us to probe for the role of Sp4 in regulating any or all of COX subunit genes in primary neurons as well as in N2a cells, a cell type commonly used by investigators of neurons. The goal of the present study is to use multiple approaches to elucidate this issue.

## Materials and methods

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996), and all protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee (approval can be provided upon request). All efforts were made to minimize the number of animals used and their suffering. The ARRIVE guidelines have been followed.

### Cell culture

Mouse N2a neuroblastoma cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Sigma, St Louis, MO), penicillin (50 units/ml), and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator supplied with 5% CO<sub>2</sub>.

Primary visual cortical neurons from 1-day-old mice (C57Bl/6, both sexes, Jackson Laboratory, Bar Harbor, ME) were cultured according to methods described previously for rats (Ongwijitwat and Wong-Riley, 2004). The density of plating in 35 mm poly-L-lysine-coated dishes was ~200,000 cells/dish. The culture media contained Neurobasal-A together

with B27 supplement (Invitrogen). Glial cell proliferation was minimized by the addition of Ara-C (Sigma) to the media.

### ***In Silico* analysis of Sp4 binding**

The NCBI mouse genome database was used to determine DNA sequences 1 kb upstream and 1 kb downstream of the transcription start point (TSP) for all 10 nucleus-encoded *COX* subunit genes as well as for *TFAM*, *TFB1M*, and *TFB2M* genes. The Sp binding motif of 'GGGCGG' and its complement 'CCGCC', or the atypical motif of 'CACCC' and its complement 'GGGTG', was searched for the promoter of each gene.

NCBI's Ensembl interface was used to align promoter sequences for the conservation of Sp binding motif in mice, rats, and humans.

### ***In vivo* analysis of Sp4 binding by chromatin immunoprecipitation (ChIP) assays**

Chromatin immunoprecipitation assays were done according to methods described previously (Priya et al., 2013a). Approximately 0.1 g of visual cortical tissue was obtained from fresh murine brains and fixed with 2% formaldehyde at 24°C for 20 min. They were then placed in a swelling buffer (85 mM KCl, 5 mM PIPES, pH 8.0, protease inhibitors, and 1% NP-40), homogenized, centrifuged to obtain the nuclei, and sonicated. The lysate was then immunoprecipitated with either the control anti-NGFR (nerve growth factor receptor) p75 goat polyclonal antibodies (2 µg) (C20, sc-6188, Santa Cruz Biotechnology, Santa Cruz, CA) or Sp4 polyclonal rabbit antibodies (2 µg) (V20, sc-645, Santa Cruz Biotechnology). No antibody served as another immunoprecipitation control. Precipitated chromatin was used in a PCR with primers that encompassed putative SP binding sites identified *in silico* (Table 1). -actin exon 5 was the negative control and murine NT3 promoter with known Sp4 binding site (Ramos et al. 2009) was the positive control (see Table 1). DreamTaq polymerase (Thermo-Fisher Scientific, Waltham, MA, USA) was used with cycling parameters as follows: hot-start at 94°C for 120 s followed by denaturation at 94°C for 30 s, annealing at 59.5°C for 30 s, and extension at 72°C for 20 s (with 32–36 cycles per reaction). The addition of betaine improved the quality of PCR. Products were run on 2% agarose gels and visualized with ethidium bromide.

### **Effect of Sp4 silencing and depolarizing stimulation with KCl**

Two *Sp4* siRNA sequences (5'-CTGGACAACAGCAGATTATTA-3' and 5'-CCAGTAACAATCACTAGTGTT-3') were chosen from the RNAi Consortium's Public TRC Cloning Database at the Broad Institute and cloned into the pLKO.1 TRC cloning vector (Plasmid 10878, addgene, Cambridge, MA, USA). The pLKO.1 non-mammalian shRNA control vector, which contains a scrambled shRNA sequence that targets no known mammalian genes, was used as the negative control (SHC002, Sigma). An enhanced green fluorescent protein-containing vector (eGFP) was used to identify transfected primary neurons or N2a cells. Primary cultures of murine visual cortex neurons were plated at a density of  $2 \times 10^5$  cells/dish, and N2a cells at  $5 - 8 \times 10^6$  cells/dish. Primary neurons were co-transfected 5 days post-plating with 0.5 µg of eGFP vector and either 2 µg of shRNA plasmids (two *Sp4* sequences at equal amounts) or with 2 µg of control scrambled vectors using Neurofect (Dhar and Wong-Riley, 2011). In another set of experiments, primary neurons were transfected as above with 0.5 µg of eGFP and 2 µg of *Sp1* shRNA plasmids (Santa Cruz Biotechnology) or with 2 µg of control scrambled vectors using Neurofect. N2a cells were co-transfected the day after plating with 1 µg of eGFP and either 3 µg of the shRNA plasmids or 3 µg of the control scrambled vector using Lipofectamine 2000 (Ongwijitwat and Wong-Riley, 2006). On the day after transfection, puromycin was added to the culture medium (at a final concentration of 0.5 µg/ml for primary neurons or 5 µg/ml for N2a cells) for transfection selection. Transfection efficiency monitored by eGFP was

40% to 60% for primary neurons and 40% to 75% for N2a cells. However, puromycin selection effectively yielded 100% of transfected cells. Transfected N2a cells were further stimulated for 5 h with 20 mM KCl as described previously (Liang and Wong-Riley 2006; Yang *et al.*, 2006). Cells were then harvested for either western blot or RNA isolation.

### Effect of *Sp4* over-expression and impulse blockade with TTX

The Gateway Multisite Cloning kit (Invitrogen) was used to clone the human *Sp4* cDNA (Open Biosystems, Lafayette, CO) into the pcDNA-Dest40 vector according to the manufacturer's instructions and as described previously (Johar *et al.* 2012). The vector contains neomycin resistance that is selectable with Geneticin in mammalian cells. Primary neurons from mouse visual cortex or N2a cells were plated in 35-mm dishes at the densities described above. Primary neurons were co-transfected 5 days post-plating with 0.5  $\mu$ g of the eGFP vector and either 1.5  $\mu$ g of *Sp4* expression plasmids or 1  $\mu$ g of pLKO.1 empty vector controls by means of Neurofect. N2a cells were co-transfected the day after plating with the above vectors by means of Lipofectamine. On the day after transfection, puromycin at a final concentration described above was added to cells transfected with the control empty vectors, whereas Geneticin (Invitrogen) was added to cells transfected with *Sp4* expression vectors (at a final concentration of 100  $\mu$ g/ml for primary neurons or 250  $\mu$ g/ml for N2a cells) for transfection selection. Transfection efficiency monitored with eGFP was from 40% to 60% for primary neurons and 40% to 75% for N2a cells. However, puromycin or Geneticin selection resulted in 100% transfected cells. *Sp4*-transfected N2a cells were then subjected to impulse blockade with tetrodotoxin (TTX; 0.4  $\mu$ M) for 3 days, harvested, and processed for protein and RNA isolation.

### RNA isolation, cDNA synthesis, and real-time quantitative PCR

RNeasy kits (Qiagen, Valencia, CA) were used to isolate total RNA, which then underwent DNase I treatment and phenol-chloroform purification. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA, and the Cepheid Smart Cyclor Detection system (Cepheid, Sunnyvale, CA) was used to perform real-time quantitative PCRs, together with IQ Sybr Green SuperMix (170-8880, BioRad). Table 2 shows the primer sequences. PCR cycling parameters were: 2 min hot start at 95°C followed by 15–30 cycles of 10 s denaturation at 95°C, 15 s annealing at the  $T_m$  of each primer, and 10 s extension at 72°C. The purity of single PCR product was verified by the melt curve analysis.  $\beta$ -actin and GAPDH were used as internal controls, and the relative amount of mRNAs was determined by the  $2^{-CT}$  method (Livak and Schmittgen 2001).

### Western blot assay

Proteins from whole extracts of mouse brains, cultured mouse primary neurons, N2a cells, *Sp4* shRNA, and over-expression samples plus the appropriate controls were loaded onto 10% SDS-PAGE gel and transferred electrophoretically onto polyvinylidene difluoride membranes (Bio-Rad). After blocking, primary antibodies against Sp1 (1:1000; H-225, sc-14027, Santa Cruz Biotechnology), Sp4 (1:1000; V-20, sc-645, Santa Cruz Biotechnology), COX1 (1:1000; Molecular Probes, Life Technologies) and primarily COX4i1 (1: 1000) (Hevner and Wong-Riley 1989) were applied to the blots.  $\beta$ -actin (1:3000; Sigma) was the loading control. Sp1 polyclonal antibodies were raised in rabbits against amino acids 121–345 near the N-terminus of human Sp1, whereas Sp4 polyclonal antibodies raised in rabbits were against a proprietary region within the last 50 amino acids (734–784) at the C-terminus of human Sp4. When these two antigenic sites were compared, there were only 2 consecutive amino acids that were similar between them. When the last 50 amino acids in Sp4 were compared to the entire Sp1 protein, no homology was found. Likewise, no homology was found between N-terminus 121–345 amino acids and the entire Sp4 protein. Human Sp1 and Sp4 proteins share 51% homology, whereas mouse Sp1 and

Sp4 share 54% homology, almost all of which are located at scattered intervals between amino acids 430 and 730. On the other hand, there were 95% homology between human and mouse Sp1 and 97% homology between human and mouse Sp4. Sp4 antibodies used in the present study (V-20, sc-645) showed no band in western blots of brain nuclear extracts from *Sp4* knockout mice (Göllner et al., 2001), indicating no cross-reactivity with other proteins.

After incubating in goat-anti-rabbit or goat-anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA, USA), blots were treated with the ECL reagent (Pierce, Rockford, IL, USA) and exposed to autoradiographic film (RPI, Mount Prospect, IL, USA). Relative changes were quantified with an Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

### Cytochrome c oxidase histochemistry

Relative activity of cytochrome c oxidase was assayed with histochemistry followed by single neuron optical densitometry (Wong-Riley, 1979) on N2a cells of control, KCl- or TTX-treated, and KCl-plus-Sp4 shRNA-transfected or TTX-plus-Sp4 over-expression-transfected samples. The protocols for KCl, TTX, and transfection were the same as described above.

### Statistical analysis

Analysis of variance (ANOVA) was used to determine significance among group means, and Student's *t*-tests determined significance between two groups. Significance was set at a *P*-value of 0.05.

## Results

### Sp4 is expressed at a higher level in primary neurons and brain than in N2a cells

Western blots of proteins obtained from visual cortices of murine brains, cultured mouse primary neurons (from the visual cortex), and N2a cells are shown in Figure 1A. Sp4 was present in greater amount in brain and primary neurons than in N2a cells, whereas Sp1 was present in greater amount in N2a cells than in brain or primary neurons ( $P < 0.001$  for all) (Fig. 1B). When cultured primary neurons were stimulated with depolarizing KCl or with an impulse blocker TTX, the level of Sp4 was significantly up- or down-regulated, respectively (Fig. 1C, D).

### *In vivo* binding of Sp4 to promoters of all 10 nuclear COX subunits and 3 mitochondrial transcription factors

Promoters of *TFAM*, *TFB1M*, *TFB2M*, and all ten *COX* subunit genes were analyzed for possible *in vivo* Sp4 binding by means of chromatin immunoprecipitation (ChIP) assays. The positive control was *NT3* with a known Sp4 binding site and the negative control was *-actin* exon 5 (*Actb*). Anti-nerve growth factor receptor (NGFR) p75 antibodies served as the negative control for immunoprecipitation with the same stock of cell lysate derived from mouse visual cortical tissue. An additional no antibody control was also used. The efficiency of PCR was indicated by a 0.5% dilution of input chromatin obtained before immunoprecipitation.

Immunoprecipitation with Sp4 antibodies and amplifications with semi-quantitative PCRs revealed positive bands for the three mitochondrial transcription factors, *TFAM*, *TFB1M*, and *TFB2M*, as well as for the ten nucleus-encoded *COX* subunit genes (*4i1*, *5a*, *5b*, *6a1*, *6b*, *6c*, *7a2*, *7b*, *7c*, and *8a*) (Fig. 2). The positive control *NT3* also showed a distinct band, whereas the negative control *-actin* (*Actb*) did not. The immunoprecipitation control NGFR



(NGFR lanes) also yielded no detectable band, neither did the no-antibody control (Blank lanes) (Fig. 2).

### Knockdown of *Sp4* reduces the levels of all thirteen *COX* subunits

Transfection of N2a cells with *Sp4* shRNA led to a 67% decrease in their *Sp4* protein ( $P < 0.001$ , Fig. 3A, B) and a 70% decrease in their *Sp4* message ( $P < 0.001$ , Fig. 3C). The levels of *TFAM*, *TFB1M*, and *TFB2M* as well as of the 3 mitochondrial-encoded *COX1*, *COX2*, and *COX3* subunits were significantly reduced ( $P < 0.001$  for all, Fig. 3C upper panel). Relative to empty vector controls, the message of a COX assembly factor, surfeit 1 (*SURF1*), with a reported Sp factor binding site (Cole and Gaston, 1997) was also down-regulated ( $P < 0.001$ , Fig. 3C upper panel), as were those of 10 nucleus-encoded *COX* subunit genes (*COX4i1* to *COX8a*;  $P < 0.001$  for all, Fig. 3C, lower panel). *Sp4* knockdown also caused a 53% and 40% reduction in COX1 and COX4i1 proteins, respectively ( $P < 0.001$  for both, Fig. 3A, B).

In cultured primary neurons transfected with *Sp4* shRNA, *Sp4* mRNA levels were reduced by 71% ( $P < 0.001$ , Figure 3D). Likewise, the mRNA levels of all 13 *COX* subunits, the three mitochondrial transcription factors, and *SURF1* were significantly reduced by 65 – 75% (except for a 47% reduction in *COX 4i1*;  $P < 0.001$  for all, Fig. 3D) as compared to neurons transfected with scrambled vectors. Knocking down *Sp4* induced a slight but significant increase in the expressions of *Sp1* ( $P < 0.05$ ); likewise, knocking down *Sp1* caused a slight but significant increase in *Sp4* transcripts ( $P < 0.05$ ) (Fig. 3D). However, silencing of *Sp1* had no significant effect on the expressions of any of the 13 *COX* subunit genes, nor those of *TFAM*, *TFB1M*, *TFB2M*, or *SURF1* genes in primary neurons (Fig. 3D).

The greater reduction of transcript levels in primary neurons than in N2a cells could be related to the fact that N2a cells have a greater abundance of *Sp1* than primary neurons and, therefore, could support a higher target gene expression with *Sp4* knockdown.

### Over-expression of *Sp4* up-regulates all thirteen *COX* subunits

In N2a cells transfected with *Sp4* expression vectors, there was a 10-fold increase in *Sp4* protein ( $P < 0.001$ , Fig. 4A, B) and a 77-fold increase in *Sp4* message ( $P < 0.001$ , Fig. 4C). The message levels of *COX1*, *COX2*, *COX3*, *TFAM*, *TFB1M*, *TFB2M* and *SURF1* were all significantly up-regulated ( $P < 0.001$  for all, Fig. 4C upper panel), as were those of the 10 nucleus-encoded *COX* subunits ( $P < 0.001$  for all, Fig. 4C lower panel). Over-expression of *Sp4* also led to a 68% increase in the protein level of COX1 ( $P < 0.001$ , Fig. 4A) and a 78% increase in that of COX4i1 ( $P < 0.01$ , Fig. 4A, B).

In cultured primary neurons transfected with *Sp4* expression vectors, a 28-fold increase in their *Sp4* message was noted ( $P < 0.001$ , Fig. 4D). There was also a significant increase in their transcript levels of the 3 mitochondrial *COX* subunits, 3 mitochondrial transcription factors, *SURF1*, and 10 nucleus-encoded *COX* subunits relative to empty vector controls ( $P < 0.01 - 0.001$  for all, Fig. 4D).

### Regulation of *COX* expression by *Sp4* is related to neuronal activity

To elucidate the role of *Sp4* in activity-dependent expressions of *COX* subunit genes, N2a cells were transfected with either *Sp4* shRNA or scrambled vectors before being stimulated by 20 mM KCl-induced depolarization for 5 hours. Such KCl treatment was found to be effective in up-regulating gene expression of *COX* in primary neurons (Liang and Wong-Riley, 2006; Yang et al. 2006). Indeed, depolarizing stimulation led to a significant increase in the mRNA levels of *Sp4*, 3 mitochondrial *COX* subunits, 3 mitochondrial transcription

factors, *SURF1*, and 10 nucleus-encoded *COX* subunits as compared to scrambled vector controls ( $P < 0.001$  for all, Fig. 5A). However, in the presence of *Sp4* shRNA, all of these target genes failed to respond to KCl stimulation ( $P < 0.001$  for all as compared to scrambled vector controls and to control + KCl) (Fig. 5A).

At a concentration of 0.4  $\mu\text{M}$ , the impulse blocker TTX has been found to reduce *COX* subunit transcript levels and COX enzyme activity both *in vivo* and in primary neurons (Hevner and Wong-Riley 1993; DeYoe *et al.* 1995; Liang and Wong-Riley 2006). To determine if over-expressing *Sp4* would rescue the down-regulation of *COX* subunits imposed by impulse blockade, N2a cells were transfected with either *Sp4* expression vectors or empty vector controls were treated with 0.4  $\mu\text{M}$  TTX for 3 days. As shown in Fig. 5B, TTX alone reduced the mRNA levels of *Sp4* ( $P < 0.001$ ), 3 mitochondrial *COX* subunits ( $P < 0.001$  for all), 3 mitochondrial transcription factors ( $P < 0.001$  for *TFAM* and *TFB1M*;  $P < 0.05$  for *TFB2M*), *SURF1* ( $P < 0.05$ ), and 10 nucleus-encoded *COX* subunits ( $P < 0.001$  for *COX5a*, *6a1*, *7a2*, *7c*, and *8a*;  $P < 0.01$  for *COX4i1*, *5b*, *6b*, and *7b*;  $P < 0.05$  for *COX6c*). Thus, there was an overall suppressive effect of TTX on all *COX* subunit gene expression in neurons. On the other hand, *Sp4* over-expression rescued TTX-induced reductions in the mRNA levels of all of the above genes tested ( $P < 0.001$  for all as compared to empty vector controls and to control + TTX) (Fig. 5B).

The effect of knockdown or over-expression of *Sp4* on COX enzyme activity was tested in primary neurons. As shown in Figs. 5C and 5D, *Sp4* shRNA prevented the up-regulation of activity by KCl depolarization, whereas *Sp4* over-expression rescued COX enzyme activity from being down-regulated by TTX blockade.

### Conservation of Sp4 binding sites among mice, rats, and humans

The functional Sp4 binding sites on 10 nucleus-encoded *COX* subunits, *TFAM*, *TFB1M*, and *TFB2M* are conserved among humans, mice, and rats (data not shown). The homology ranged between 60 to 100% (mainly between 80 to 90%). As Sp1 and Sp4 recognize and bind to the same *cis*- motif, the sites for Sp4 are consistent with our recently published sites for Sp1 (Dhar *et al.*, 2013).

### Discussion

In the past, Sp1 has been the focus of most studies on Sp factors. Analysis of *COX* subunit genes have pointed to the existence of Sp1 binding sites without consideration for Sp4's potential role in regulation (Grossman and Lomax, 1997; Ongwijitwat and Wong-Riley, 2004). The present study, however, documents by means of multiple approaches that in primary neurons, it is Sp4, and not Sp1, that plays a more prominent role in regulating all 13 subunits of COX. Sp4 is more abundant in both mouse visual cortex and cultured primary neurons than in N2a cells, whereas the reverse is true for Sp1, a more ubiquitously expressed member of the Sp family. Despite its abundance in neurons, Sp4 has received very little attention in the past. The present study revealed that Sp4 directly regulates all ten nucleus-encoded *COX* subunit genes and indirectly controls the expression of three mitochondria-encoded *COX* genes by regulating the three transcription factors (*TFAM*, *TFB1M* and *TFB2M*) critical in the transcription and replication of mitochondrial DNA. Sp4 also regulates an important *COX* assembly factor *SURF1* in neurons. Significantly, knocking down *Sp4* prevented the up-regulation of COX message, protein, and activity induced by depolarizing stimulation of neuronal activity, and over-expression of *Sp4* rescued COX message, protein, and activity from being down-regulated by neuronal impulse blockade with TTX. The effect of *Sp4* silencing is much greater in primary neurons than in N2a cells.

Neurons depend almost solely on oxidative metabolism for their energy supply, and COX plays a critical role in this process, as it carries oxidative metabolism to completion and helps in the generation of ATP by actively pumping H<sup>+</sup> from the inside to the outside of mitochondria in setting up the proton gradient (Kadenbach *et al.* 1983; Wong-Riley 1989; 2012). Mitochondria-encoded subunits of COX (COX1, COX2, and COX3) are the largest and form the catalytic core of the enzyme (Kadenbach *et al.* 2000; Napiwotzki *et al.* 1997). Functions of the 10 nucleus-encoded COX subunits are beginning to be explored. They reportedly include the regulation of the catalytic activity, assembly, or stability of the enzyme (Kadenbach *et al.* 2000; Hüttermann *et al.* 2001; 2003). At high ATP/ADP ratio in the mitochondria, COX4 binds to ATP and allosterically inhibits enzyme activity (Arnold and Kadenbach 1997; Napiwotzki *et al.* 1997). Likewise, COX5b and COX6a regulate COX activity according to the ATP/ADP ratio (Frank and Kadenbach 1996; Bender and Kadenbach 2000). COX5a can bind to 3,4-diiodothyronine (T2) and relieve the intramitochondrial ATP-induced allosteric inhibition of COX activity (Arnold *et al.* 1998). COX6b assists in stabilizing the dimeric COX enzyme, and COX6c binds to cytochrome C with low affinity (Tsukihara *et al.* 1996). COX6a, 7a, and 8 have isoforms that are tissue-specific that differentially regulate COX activity (Kadenbach *et al.* 1990; 2000). The precise functions of COX subunits 7a, 7b, 7c, and 8a are not fully understood at this time.

In the Sp family of transcription factors, Sp1, Sp3, and Sp4 are structurally-related zinc finger proteins. These factors recognize the same GC-rich sequences in CpG islands and bind with similar affinity; hence, they compete or cooperate with each other. To date, at least 12,000 Sp-binding sites have been found in the human genome, most of which have been associated with genes involved in many cellular functions (Safe and Abdelrahim 2005; Suske 1999; Li and Davie 2010). Sp1 and Sp3 are ubiquitously expressed, whereas Sp4 is expressed mainly in neurons and testes (Suske 1999; Wierstra 2008). Sp4 has both activating and repressing domains for transcription, and both are operating in neurons (Suske 1999; Wierstra 2008). *Sp1* knockout mouse embryos die at about 10 days of gestation (Marin *et al.*, 1997), whereas *Sp4* knockout mice survive up to birth. After birth, however, two-thirds of them die within 4 weeks, and surviving ones have abnormalities in their reproductive system and reproductive behavior (Göllner *et al.*, 2001). Thus, physiological functions are not identical between these two Sp factors. Once neurons are differentiated and become postmitotic, Sp4 exerts transcriptional repression on genes involved in mitosis, and the expression of Sp1, which regulates many mitotic proteins, is down-regulated (Mao *et al.* 2006; 2007; 2009). Recently, we found that in N2a cells, Sp1 regulates the expression of all thirteen *COX* subunit genes (Dhar *et al.* 2013). However, as shown in the present study, Sp4 plays a more dominant role in regulating *COX* in primary neurons. Knocking down *Sp1* caused a slight but significant increase in the expression of *Sp4* in primary neurons, and vice versa. However, whereas silencing *Sp4* is detrimental to the expressions of all *COX* subunit genes, suggesting that the slight increase in *Sp1* expression was not sufficient to rescue these target genes in primary neurons, silencing *Sp1* essentially had no effect on the target genes, indicating that they are regulated mainly by Sp4 and not by Sp1 in primary neurons.

Moreover, we recently found that in primary neurons, only Sp4 and not Sp1 regulates the expression of key glutamatergic NMDA receptor *Grin1*, *Grin2a*, and *Grin2b* genes (Priya *et al.* 2013b). Thus, Sp4 is able to co-regulate mediators of energy metabolism (COX) as well as mediators of synaptic transmission (NMDA receptor subunit genes), thereby enabling the coupling of energy metabolism and neuronal activity at the transcriptional level. This attests to the critical role of Sp4 in normal neuronal functioning.

Our earlier studies have shown that levels of COX transcripts, proteins, and activity are regulated by neuronal activity (Wong-Riley 1989; Hevner and Wong-Riley 1993; DeYoe *et*



*al.* 1995; Wong-Riley *et al.* 1998; Liang and Wong-Riley 2006; Yang *et al.* 2006). The present study indicates that Sp4 itself is also regulated by neuronal activity. Sp4 message and protein are up-regulated by depolarizing stimulation and down-regulated by impulse blockade. Thus, when Sp4's own expression is knocked down by shRNA, it can no longer support a heightened level of target gene transcription induced by KCl stimulation. Likewise, when Sp4 is over-expressed, it can rescue its target gene expression suppressed by TTX blockade. Thus, Sp4 mediates the activity-dependent regulation of all 13 *COX* subunit genes, the mitochondrial transcription factor genes, and *SURF1* gene in neurons. In mature neurons, when the level of Sp1 is normally reduced (Mao *et al.* 2007; present study), Sp4 becomes the dominant Sp factor.

COX is a multisubunit, bigenomic, and multichromosomal protein whose transcriptional regulation presents a true operational challenge. To achieve a fine-tuned regulation in neurons, multiple transcription factors are likely involved and a well-orchestrated mechanism must be in place. Besides Sp4, neurons also rely on NRF-1 and NRF-2 to regulate all 10 of its nucleus-encoded subunit genes (Ongwijitwat and Wong-Riley 2005; Ongwijitwat *et al.* 2006; Dhar *et al.* 2008). These transcription factors also regulate *TFAM*, *TFB1M*, and *TFB2M*, thereby controlling the expression of the 3 mitochondrial *COX* subunit genes (Virbasius and Scarpulla 1994; Gleyzer *et al.* 2005; Dhar *et al.* 2013). Remarkably, the 10 nucleus-encoded *COX* subunit genes and genes for *TFAM*, *TFB1M*, and *TFB2M* are all transcribed in the same dynamic transcription factory (Dhar *et al.* 2009).

The involvement of multiple transcription factors in regulating the same set of genes (13 in the case of COX) necessitates one of three possible mechanisms: complementary (each factor regulating complementary sets of target genes), concurrent and parallel (all regulating the same set of target genes in the same positive or negative direction), or a combination of complementary and concurrent/parallel. NRF-1 and NRF-2 have previously been found to operate in a concurrent and parallel manner in regulating the same set of genes in neurons (Ongwijitwat and Wong-Riley, 2005; Dhar and Wong-Riley, 2008, 2009; Priya *et al.* 2013a). The present study revealed that Sp4 operates in a concurrent and parallel mechanism with NRF-1 and NRF-2 in regulating all 13 COX subunit genes in neurons. As the binding sites for Sp4, NRF-1, and NRF-2 can be in close proximity to one another for a number of COX subunit genes (our unpublished observations), it is possible that they may interact with each other while regulating their target genes in neurons. The verification or dismissal of such interactions awaits future investigation. Besides transcription factors, coactivators such as peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) are likely to be involved in the regulation of *COX* in neurons. PGC-1 powerfully coactivates NRF-1 and NRF-2 and is a master regulator of mitochondrial biogenesis (Wu *et al.* 1999; Scarpulla 2008). It responds to changes in neuronal activity earlier than those of NRF-1 and NRF-2 (Liang and Wong-Riley 2006; Liang *et al.* 2010). Future studies will determine if PGC-1 interacts directly with Sp4 in neurons.

## Acknowledgments

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## Abbreviations

<b>ChIP</b>	chromatin immunoprecipitation
<b>COX</b>	cytochrome c oxidase
<b>EMSA</b>	electrophoretic mobility shift assay

<b>NRF-1</b>	nuclear respiratory factor 1
<b>NRF-2</b>	nuclear respiratory factor 2
<b>PGC-1</b>	peroxisome proliferator-activated receptor- coactivator 1
<b>Sp1</b>	specificity protein 1
<b>Sp3</b>	specificity protein 3
<b>Sp4</b>	specificity protein 4
<b>Surf1</b>	Surfeit locus protein 1
<b>TFAM</b>	transcription factor A of mitochondria
<b>TFB1M</b>	transcription factor B1 of mitochondria
<b>TFB2M</b>	transcription factor B2 of mitochondria
<b>TSP</b>	transcription start point

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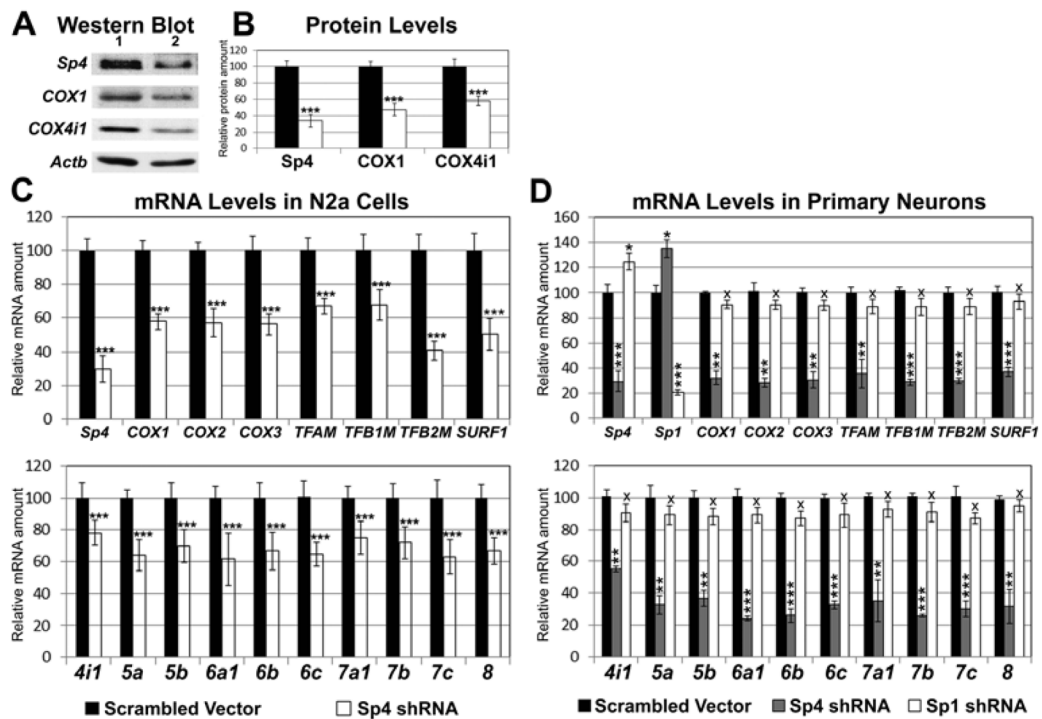
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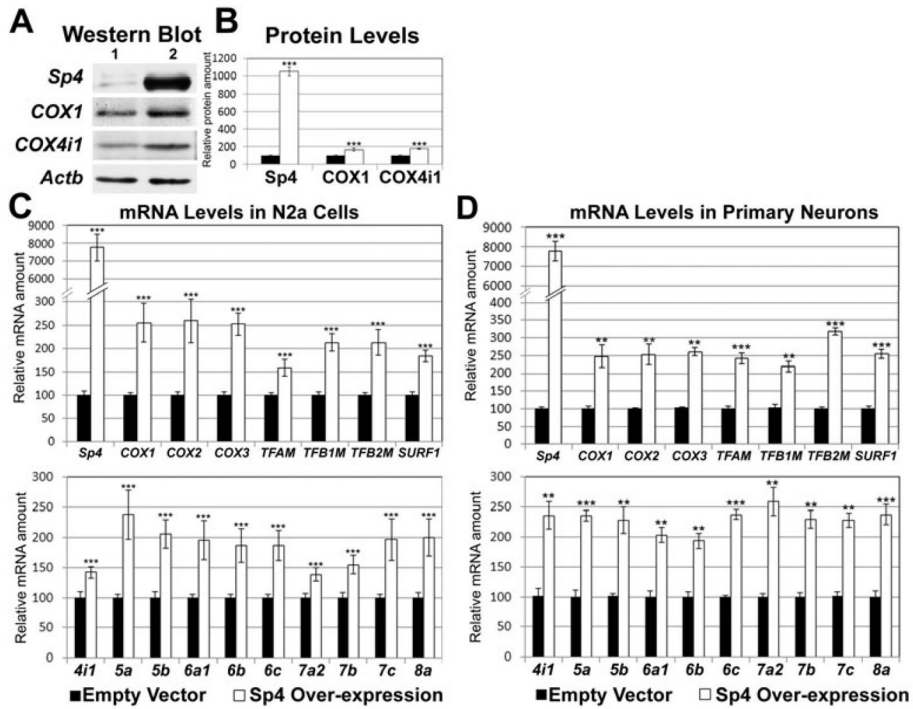
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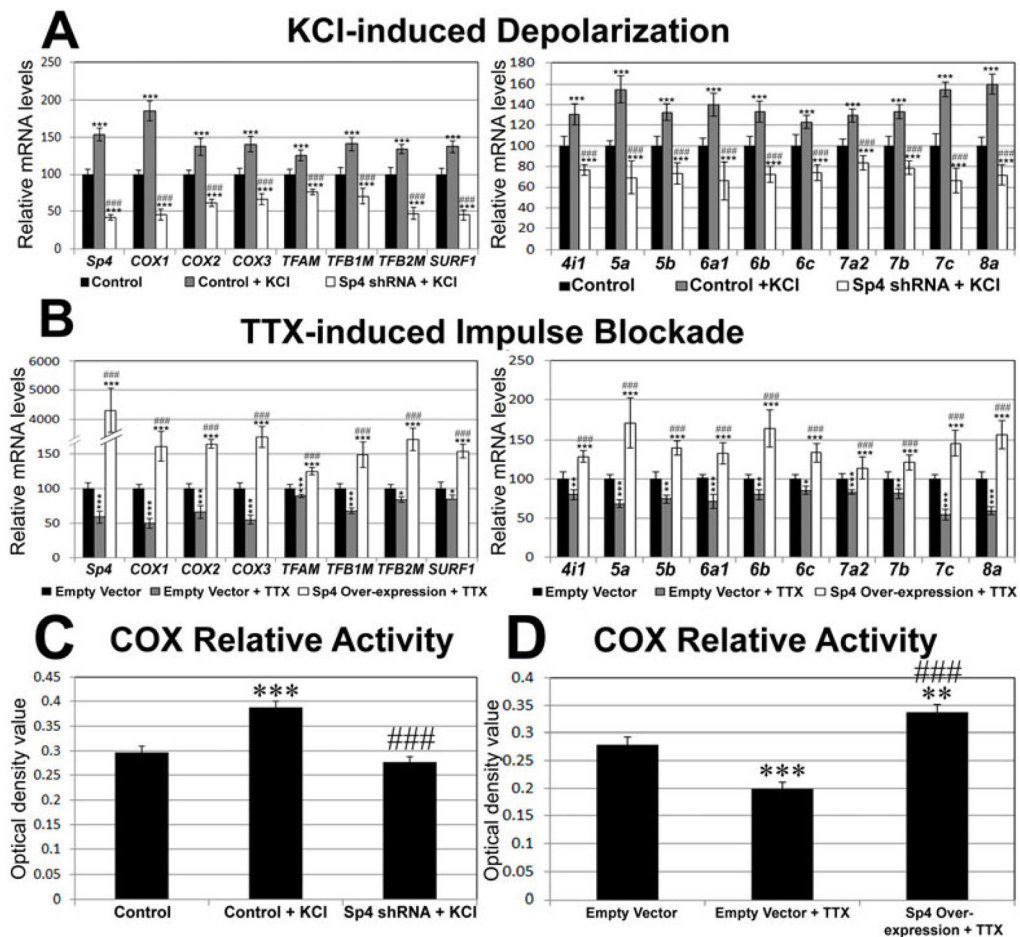




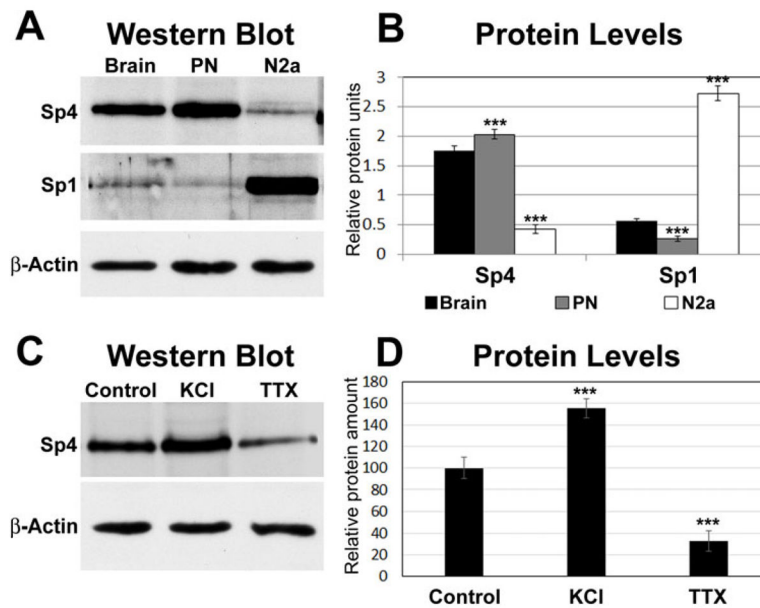
**Fig. 1.** Mouse brain (visual cortex) and cultured primary neurons (from mouse visual cortex) express more Sp4 than N2a cells. **A**, Western blots of proteins obtained from mouse brain, cultured mouse primary neurons (PN), and N2a cells labeled with Sp1 or Sp4 antibodies. actin served as the loading control. N = 3 for each group. **B**, Quantification of bands indicated that both brain and primary neurons have much more Sp4 than N2a cells. On the other hand, N2a cells have significantly more Sp1 than either brain or primary neurons. **C**, Western blots of Sp4 proteins from cultured primary neurons subjected to KCl or TTX. actin was the loading control. N = 3 for each group. **D**, Quantification of bands indicated that Sp4 protein was up-regulated by KCl depolarizing stimulation and down-regulated by TTX impulse blockade as compared to controls. \*\*\*=  $P < 0.001$ .



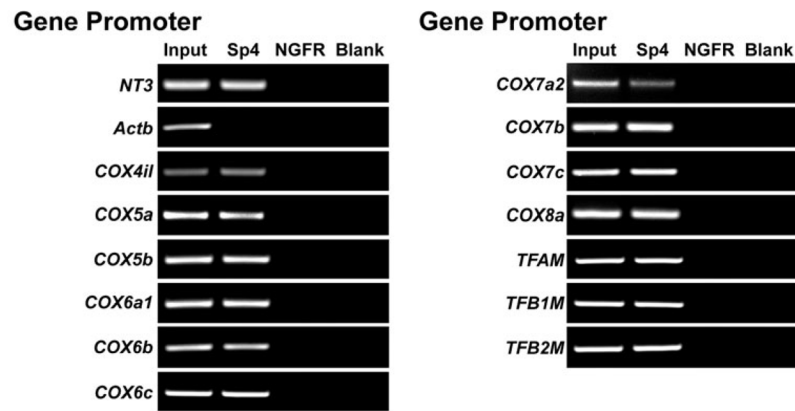
**Fig. 2.** Sp4 binds to the promoters of all 10 nucleus-encoded *COX* subunits as well as *TFAM*, *TFB1M*, and *TFB2M* *in vivo*. ChIP assays were done on mouse visual cortical tissue immunoprecipitated with anti-Sp4 antibodies (Sp4 lanes), anti-nerve growth factor receptor p75 antibodies (negative control, NGFR lanes), or no antibody (negative control, Blank). Input lanes indicate 0.5% of input chromatin without immunoprecipitation. *NT3* promoter served as the positive control and *-actin (Actb)* served as the negative control for Sp factors. Promoters of all 10 nucleus-encoded *COX* subunit, *TFAM*, *TFB1M*, and *TFB2M* were immunoprecipitated with anti-Sp4 antibodies and not with anti-NGRF or without exogenous antibodies.



**Fig. 3.** Silencing of *Sp4* in N2a cells (A–C) or in primary neurons (D) suppressed expression of all thirteen COX subunits. **A**, Down-regulation of *Sp4*, *COX1*, and *COX4i1* protein levels in *Sp4*-silenced cells as compared to those transfected with scrambled vector controls. Lane 1, scrambled vectors; lane 2, *Sp4* shRNA. -actin (*Actb*) served as a loading control. N = 6 for each group. **B**, Quantification of protein levels shown in A. Solid bars, controls; open bars, *Sp4* shRNA. **C**, *Sp4* shRNA-transfected N2a cells had a significant down-regulation of transcript levels for *Sp4*, the mitochondrial-encoded *COX* subunits (*COX1*, *COX2*, and *COX3*), mitochondrial transcription factors (*TFAM*, *TFB1M*, and *TFB2M*), *SURF1*, and nucleus-encoded *COX* subunits (*COX4i1* - *COX8a*) as compared to those transfected with scrambled vectors. N = 5 for each data point; \*\*\*=  $P < 0.001$  as compared to scrambled vectors. **D**, *Sp4* shRNA-transfected primary neurons also had a significant down-regulation of *Sp4*, mitochondrial encoded *COX* subunits (*COX1*, *COX2*, and *COX3*), mitochondrial transcription factors (*TFAM*, *TFB1M*, and *TFB2M*), *SURF1*, and nucleus-encoded subunits (*COX4i1* - *COX8a*) compared with scrambled vector controls. Silencing of *Sp4* induced a slight but significant increase in the expressions of *Sp1*. Silencing of *Sp1* also induced a slight but significant increase in the transcripts of *Sp1*. However, *Sp1* shRNA did not lead to any change in the expressions of all 13 *COX* subunit genes, nor those of *TFAM*, *TFB1M*, *TFB2M*, and *SURF1* in primary neurons. N = 3 for each data point; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ . X = Non-significant as compared to scrambled vector controls.

**Fig. 4.**

Over-expression of *Sp4* in N2a cells (A – C) or in primary neurons (D) increased expression of all thirteen COX subunits. **A**, Up-regulation of Sp4, COX1, and COX4i1 protein levels in cells transfected with Sp4 expression vectors as compared to empty vector controls. Lane 1, empty vectors; Lane 2, *Sp4* over-expression. N = 6 for each group. **B**, Quantification of protein levels shown in A. Solid bars, controls; open bars, *Sp4* over-expression. **C**, *Sp4* over-expression led to a significant up-regulation of *Sp4*, mitochondrial encoded COX subunits (*COX1*, *COX2*, and *COX-3*), mitochondrial transcription factors (*TFAM*, *TFB1M*, and *TFB2M*), *SURF1*, and nucleus-encoded COX subunits (*COX4i1* - *COX8a*) as compared to empty vector controls. N = 6 for each group. All \* *P* values were compared to empty vectors. \*\* *P* < 0.01 and \*\*\* *P* < 0.001 when compared to empty vectors. **D**, *Sp4* over-expression in primary neurons also led to a significant up-regulation of *Sp4*, mitochondrial encoded COX subunits (*COX1*, *COX2*, and *COX-3*), mitochondrial transcription factors (*TFAM*, *TFB1M*, and *TFB2M*), *SURF1*, and all 10 nucleus-encoded subunits (*COX4i1* - *COX8a*) compared to empty vector controls. N = 3 for each group. All \* *P* values were compared to empty vectors. \*\* *P* < 0.01 and \*\*\* *P* < 0.001.



**Fig. 5.** Sp4 and its target genes are all regulated by neuronal activity in N2a cells. **A.** KCl-induced depolarization significantly increased mRNA levels of *Sp4*, 3 mitochondrial-encoded *COX* subunits, 3 mitochondrial transcription factors, *SURF1*, and 10 nucleus-encoded *COX* subunits. In the presence of *Sp4* shRNA, however, KCl was not able to up-regulate the levels of all transcripts. **B.** TTX-induced impulse blockade led to reduced levels of *Sp4*, 3 mitochondrial-encoded *COX* subunits, 3 mitochondrial transcription factors, *SURF1*, and 10 nucleus-encoded *COX* subunits. Over-expression of *Sp4*, however, rescued all 13 *COX* subunits and 3 mitochondrial transcription factors as well as *SURF1* from TTX-induced suppression N = 6 for each data point; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$  when compared to either scrambled (**A**) or empty vector (**B**) controls. ### =  $P < 0.001$  when compared to control + KCl (**A**) or control + TTX (**B**). **C.** Optical densitometric analysis of reaction product of cytochrome c oxidase histochemistry in N2a cells. KCl induced a significant increase in the relative activity of COX as compared to scrambled vector controls. However, no up-regulation was observed in cells transfected with *Sp4* shRNA. N for optical densitometric (OD) analysis = 122 cells for control, 125 for control + KCl, and 128 for *Sp4* shRNA + KCl. \*\*\* =  $P < 0.001$  when compared to controls. ### =  $P < 0.001$  when compared to control + KCl. No significance was found between *Sp4* shRNA + KCl and controls. **D.** Three days of TTX treatment led to significant reduction of COX relative activity. However, in N2a cells transfected with *Sp4* expression vectors, the activity was increased rather than down-regulated. N for OD analysis = 115 cells for empty vector controls, 162 for empty vector + TTX, and 207 for *Sp4* over-expression + TTX. \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$  when compared to controls. ### =  $P < 0.001$  when compared to control + KCl.



**Table 1**

Primers for ChIP assays.

Gene	Sequence	Amplicon length (bp)
<i>COX4i1</i>	F: 5 -GAAAACGTCTGCCGAAAAG-3 R: 5 -GTCACCTGCCACCGCTG-3	284
<i>COX5a</i>	F: 5 -CCACGCAGGAATGTTCACTA-3 R: 5 -ACGAGAAGCCGGTGTGAG-3	237
<i>COX5b</i>	F: 5 -GCGTTGTTAGACTCCCACCA-3 R: 5 -AGCTGGTCACGTACCTCCAG-3	200
<i>COX6a1</i>	F: 5 -GCTGACAAGCAGGGAGATG-3 R: 5 -GACGCCATCATGGAACTACA-3	215
<i>COX6b</i>	F: 5 -GCCCAGCAACAATAATAAGCA-3 R: 5 -TAGCAAAGACGCCAATGTCA-3	196
<i>COX6c</i>	F: 5 -GGTGAGAATGGTGGAGAGAGA-3 R: 5 -GCAAAATACAAGGGGAAACG-3	299
<i>COX7a2</i>	F: 5 -CGTTTGCTTCCATTGTGATT-3 R: 5 -AAACGGAACTCCCTCCTAGC-3	219
<i>COX7b</i>	F: 5 -GATGTTGCCCTTAGCCAAAA-3 R: 5 -CTAGCTTCCCTTCCAGTGA-3	194
<i>COX7c</i>	F: 5 -ACATTTCCACAATCCATCG-3 R: 5 -ATGGCCGTACCACCTAATC-3	189
<i>COX8a</i>	F: 5 -TGGGAGCAAAGTGTCTCAT-3 R: 5 -GTCCAAGGTCAGGGAGTCAA-3	213
<i>TFAM</i>	F: 5 -GTGACACAAGCCGCAGCAC-3 R: 5 -CACTACCAGCGTGGGAACTCC-3	331
<i>TFB1M</i>	F: 5 -CTGAAAGAAGTAATGGACGCG-3 R: 5 -GTGGGACCTTGGAGAAGAC-3	340
<i>TFB2M</i>	F: 5 -GAGGATCGGACACCTCTAGC-3 R: 5 -CACATTCACCACCACACTAGG-3	290
NT3	F: 5 -GAGCAAACCTCCAAAATGCCAGG-3 R: 5 -AAAGTTGCGCCGGGCTATCTC-3	198
-Actin exon 5	F: 5 -GCTCTTTTCCAGCCTTCCTT-3 R: 5 -CGGATGTCAACGTACACTT-3	187

**Table 2**

Primers for real-time quantitative PCR.

Gene	Sequence	Amplicon (bp)	T <sub>m</sub> (°C)
<i>COX1</i>	F: 5 -GCCTTGGCTTCAAAACGAGA-3	105	58
	R: 5 -GGTTGGTTCCTCGAATGTGT-3		
<i>COX2</i>	F: 5 -TCTCCCCTCTCTACGCATTC-3	161	59
	R: 5 -CAGGTTTTAGGTGCGTTTGTG-3		
<i>COX3</i>	F: 5 -ACTTCACCATCCTCCAAGC-3	151	59
	R: 5 -TGTCGTAGTAGGCAAACAATAAGG-3		
<i>COX4i1</i>	F: 5 -TCTACTCGGTGTGCTTCG-3	253	59.5
	R: 5 -ACTCATTGGTGCCCTGTTC-3		
<i>COX5a</i>	F: 5 -GGAGTTGCGTAAAGGGATGA-3	247	60
	R: 5 -CACTTTGTCAAGGCCAGTT-3		
<i>COX5b</i>	F: 5 -GGAGGTGGTGTCCCTACTGA-3	241	59.5
	R: 5 -CAGCCAGAACCAGATGACAG-3		
<i>COX6a1</i>	F: 5 -TCAACGTGTTCTCAAGTCGC-3	115	60
	R: 5 -AGGGTATGGTTACCGTCTCCC-3		
<i>COX6b</i>	F: 5 -ATGGCCGAAGACATCAAGAC-3	250	60
	R: 5 -CAGGAAATGTGCCTTCTGCT-3		
<i>COX6c</i>	F: 5 -AGCGTCTGCGGGTTCATA-3	154	60
	R: 5 -GCCTGCCTCATCTCTCAAA-3		
<i>COX7a2</i>	F: 5 -GAGGACCATCAGCACCATT-3	234	59.5
	R: 5 -TGGAGACTGGGATGACGAC-3		
<i>COX7b</i>	F: 5 -CGAAGCATTAGCAAGTGGT-3	209	59.5
	R: 5 -TGGCATGACTACTGATCTCTCC-3		
<i>COX7c</i>	F: 5 -TCTGCCTCCGTCTCTGC-3	145	60
	R: 5 -AGAAAGGAGCAGCAAATCCA-3		
<i>COX8a</i>	F: 5 -TCCTGCTTCGTGTGTGTCT-3	70	59
	R: 5 -TCCCGCTTCTGTAGCTTTC-3		
<i>TFAM</i>	F: 5 -CAGGGCTGCAATTTTCCTAA-3	141	58
	R: 5 -CCGAAGTGTTTTCCAGCAT-3		
<i>TFB1M</i>	F: 5 -AAGATGGCCCTTTCGTTTATGG-3	102	59
	R: 5 -GACTGTGCTGTTTGCTTCCTG-3		
<i>TFB2M</i>	F: 5 -CCAAAACCCATCCCGTCAAAT-3	135	59
	R: 5 -AAGGGCTCAAATGTGGAATAAA-3		
<i>SURF1</i>	F: 5 -GGTCTCTGCTTTAATCCCTGC-3	119	61
	R: 5 -GATGGGCTCAGCCATGACT-3		
<i>Sp4</i>	F: 5 -TTGCAGCAAGGCCAGCAGACC-3	130	63
	R: 5 -GCTTCTTCTTCTGTTTCACTGCT-3		
<i>NT3</i>	F: 5 -TCACCACGGAGGAAACGCTAT-3	104	61

Gene	Sequence	Amplicon (bp)	Tm (°C)
	R: 5 -TCAATGGCTGAGGACTTGTCG-3		
<i>Actb</i>	F: 5 -GGCTGTATCCCTCCATCG-3	154	59.5
	R: 5 -CCAGTTGGTAACAATGCCATGT-3		